1978

Rat Brain Synaptic Membrane Antigens Shared With Endoplasmic Reticulum

Leonard Paul Kleine

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RAT BRAIN SYNAPTIC MEMBRANE ANTIGENS SHARED WITH ENDOPLASMIC RETICULUM

BY

Leonard Paul Kleine
Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
January, 1978

A B S T R A C T

This study is an attempt to identify and characterize antigens of the synaptic membrane (RSM) of rat central nervous system neurons. Antisera were obtained by hyperimmunization of rabbits with rat RSM and brain microsomes (RBM). It is expected that antibodies elicited by the immunogens of the membrane could be highly sensitive, specific agents for the study of the structure and function of the synaptic plasma membrane complex which includes those regions of the neuronal membrane directly involved in the transmission of electrical impulses from one neuron to another.

The swelling of synaptosomes in 0.4M glycerol was inhibited by an anti-RSM serum. Absorption of the antiserum with increasing amounts of RSM or RBM indicated that most of the antibodies responsible for inhibition of synaptosome swelling (ISS) were directed against antigens shared by RSM and RBM. About 10% of the antibody activity was found to be directed specifically against synaptosomes. It was found that membranes of the RBM fraction also swell in 0.4M glycerol and thus, the presence of microsomes in the synaptosome fraction complicates the interpretation of ISS results.

The two major antigens derived from RSM, designated antigens X1 and X2, were clearly found to be identical to the two most prominent antigens of salt washed microsomes

(RBER), by immunodiffusion analyses. The antisera were absorbed with the soluble fraction of rat brain, which removed antibodies directed against antigens in the soluble fraction of brain. Antigens X1 and X2 are considered to be integral components of the synaptic membrane matrix and the microsomal membrane matrix because they were not removed from RSM or RBM by sequential washing with sodium salts, but were extracted with Triton X-100 or butanol-H_2O.

Sequential treatment of RSM and RBER with different concentrations and volumes of Triton X-100 yielded Tx1a, Tx1b and Tx2 extracts; Tx1 solutions contained antigens X1 and X2, while Tx2 solutions contained only X2. Treatment of RSM or RBER with butanol rendered both antigens soluble in water.

Quantitative immunochemical analyses demonstrated that Triton X-100 extracts similar amounts of X2 from both RSM and RBER. Gel filtration of Tx2 extracts, yielded X2 solutions that contain only 2-3 contaminants. The molecular weight of X2 was estimated to be in the order of 28-32,000 daltons.

This investigation was supported by a Medical Research Council grant and an Ontario Mental Health grant to Dr. C. F. C. MacPherson. The author was awarded Ontario Graduate Scholarships and Special Teaching Assistantships (U. W. O.). The writer wishes to express his appreaciation to these organizations for their support.

ACKNOWLEDGMENTS

I wish to express my sincerest appreciation to my supervisor, Dr. C. F. C. MacPherson, for her continued guidance, interest, and encouragement throughout the course of this investigation and for the advice in the preparation of this thesis.

I would like to thank Miss C. Th'ng, Mr. R. Romilowich and Mr. K. Weir for their excellent technical assistance and friendship. Grateful appreciation is also expressed to Miss H. Armstrong for technical assistance and help with the photography. The help of Mrs. A. Thede, Mr. P. Huie and Dr. G. Miller with the electron microscopy is gratefully acknowledged.

I would also like to thank Dr. F. Possmayer for advice and help with the enzyme assays.

Finally, I would like to thank my wife, Chia-mei, for her assistance, understanding and encouragement during the completion of this research and the writing of the thesis.

To my wife and sons, who for many nights and weekends were without husband or father.

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ABBREVIATIONS

Ach	Acetylcholine.
AChE	Acetylcholinesterase.
BSA	Bovine Serum Albumin.
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid).
IgG	Immune globulin G.
ISS	Inhibition of synaptosome swelling.
PAGE	Polyacrylamide gel electrophoresis.
PSD	Postsynatpic density.
RBE	Rat brain soluble fraction.
RBER	Rat brain endoplasmic reticulum.
RBM	Rat brain microsomes.
RSA	Relative specific activity.
RSM	Rat brain synaptic membrane.
SDH	Succinate dehydrogenase.
SDS	Sodium dodecyl sulfate.
SJC	Synaptic junctional complex.
SM	Synatpic membrane.
SV	Synaptic vesicles.
Tris	Tris (hydroxymethyl) methylamine.

CHAPTER 1. INTRODUCTION

Due to the very specialized functions of the nervous system, neurons and glia, which form a widely diverse population of cells, are among the most highly differentiated of all cell types. The synapse, which is probably the most specialized region of neural membranes, has attracted much attention from anatomists, neurophysiologists, pharmacologists and biochemists. The aim has been to gain a better understanding of synaptic function at both the cellular and molecular levels.

The immunological approach for the study of synaptic membranes (SM) has been used extensively in recent years, but the state of the art is such that few antigenic components of the synaptic membrane have been characterized. This study, thus, is an attempt at the characterization and purification of a few highly immunogenic (in rabbits) components present in the rat brain synaptic plasma membrane. Special emphasis is placed on antigens shared by SM and rat brain endoplasmic reticulum.

The rationale behind this approach is that the use of antibodies is a precise, sensitive and specific method by which to identify, quantitate and monitor the purification of membrane components that could not otherwise be studied.

The significance of such a study is that the results

could form the basis for future studies on: (1) the molecular organization of SM, (2) the comparison of SM antigens in the normal and diseased state, and (3) the effect of monospecific antibodies on memory and learning.

1.1 ISOLATION OF BRAIN SUBCELLULAR FRACTIONS

1.1.1 Isolation of Synaptosomes

Over the years subcellular fractionation has become one of the major techniques of experimental biology. This can be traced mainly to the development and refinement of a number of techniques including density gradient centrifugation and electron microscopy. Density gradient centrifugation has supplemented results obtained by rate or differential centrifugation, enabling one to obtain a larger number of defined subfractions. Electron microscopy has allowed for the morphological characterization of the subfractions obtained.

Subcellular fractionation developed mainly due to work with liver tissue by Albert Claude (1946 a and b). By the use of differential centrifugation, he was able to divide tissue homogenates into four primary fractions namely the nuclear, mitochondrial, microsomal, and soluble fractions. Since then, the enzymes of the four fractions have been enumerated and the morphology of the fractions documented by electron microscopy. Subfractions from each of the four primary ones have been obtained and characterized mainly due to the development of density gradient centrifugation, in large part due to the efforts of De Duve (1964).

The techniques developed for liver were adapted to the study of brain. Brody and Bain (1952) were among the first to use the four fraction scheme in order to study subcellular components of brain.

One of the major problems arising from the use of the four fraction scheme was that brain mitochondria were thought to differ substantially from the mitochondria of other tissues. This was mainly due to the fact that the brain mitochondrial fraction was not recognized as being very heterogeneous.

The independent analyses of the mitochondrial fraction by Whittaker and by De Robertis led to the isolation and identification of non-mitochondrial components. In 1956, Hebb and Smallman reported that more than half of the choline acetyltransferase could be found in the mitochondrial fraction (P_2). Hebb and Whittaker (1958) demonstrated that the major portion of acetylcholine (ACh) also localized in this fraction. When placed on discontinuous sucrose density gradients, the P_2 fraction could be separated into three subfractions. The fraction banding between 0.8 and 1.2M sucrose contained most of the ACh and choline acetyltransferase and morphologically was composed of vesicular and granular material with few mitochondria. Whittaker (1959) originally equated the vesicular material with synaptic vesicles which could be seen in electron micrographs of nerve endings and which were postulated to be neurotransmitter containing particles. This erroneous assumption was corrected in 1960 (Gray and Whittaker) when improved electron microscopy techniques indicated that

the acetylcholine containing fraction closely resembled the synapse or nerve ending.

It was generally accepted that " . . . the distinguishing features of the synapse are: a club-like or bag-like swelling of a fine axon termination bounded by a unit membrane; the cytoplasm within densely packed by vesicles about 300-800 Å in diameter and usually containing one or more small mitochondria; the postsynaptic membrane characteristically thickened." (Whittaker, 1965). All these features could be seen in the submitochondrial fraction and the nerve-endings as isolated were labelled synaptosomes (Whittaker, Michaelson and Kirkland, 1964).

De Robertis and co-workers arrived at similar results. They were the first to introduce the term "synaptic vesicle" (De Robertis and Bennett, 1954) and demonstrated that Whittaker's original synaptic vesicle fraction actually consisted of synaptosomes which were filled with synaptic vesicles (SV) and mitochondria (De Robertis *et al.*, 1961 a and b).

De Robertis, Pellegrino De Iraldi, Rodriguez De Lores Arnaiz and Salganicoff (1962) divided the mitochondrial fraction into five subfractions, two of which were found to contain synaptosomes. They had in fact partially separated cholinergic from non-cholinergic synaptosomes.

Originally, when brain tissue was homogenized and subjected to differential centrifugation, no one expected that a large proportion of the plasma membranes would sediment in the crude mitochondrial fraction. It has since

been well documented that upon homogenization, synaptosomes are formed from sheared or pinched off nerve endings which reseal to form vesicles (De Robertis *et al.*, 1961 a and b; De Robertis *et al.*, 1962; Gray and Whittaker, 1962; Whittaker, 1965, 1966). This, according to Whittaker (1965), results primarily from differences in mechanical and physical properties between the nerve ending and the structures which surround it. During rate or differential centrifugation, separation of the various components is due to differences in their densities, sizes, and shapes. It is therefore evident that conditions of homogenization are critically important since they will determine the size and shape of the particulate matter in homogenates. The ideal conditions for synaptosome formation have been investigated by Whittaker (1969) and by Whittaker and Dowe (1965) who reported that a clearance of 0.25 mm between pestle and homogenizer with a pestle speed of 840 rpm results in the highest yield of synaptosomes. Higher speeds or lower clearance results in a greater disruption of the nerve ending membranes and therefore in lower yield, while lower speeds will leave many cells intact.

Whittaker used centrifugal forces of 17,000g for 50-60 min in order to pellet the P_2 fraction while De Robertis used 11,500g for 20 min. In the former case, there are fewer synaptosomes in the supernatant but a large microsomal contamination of the pellet while in the latter case there are a large number of synaptosomes remaining unpelleted (Gray and Whittaker, 1962; De Robertis *et al.*, 1962). This is therefore a choice between yield or purity. A number of

reports indicate that washing of the crude mitochondrial fraction from two to five times will eliminate the majority of microsomal contamination (Jones, Mahler and Moore, 1975; Gurd, Jones, Mahler and Moore, 1974; Morgan, Wolfe, Mandel and Gombos, 1971).

Both Whittaker and De Robertis used sucrose density gradients for the subfractionation of the P_2. This seems to cause extensive damage to the osmotically sensitive synaptosomes. A number of isolation procedures have therefore replaced sucrose with Ficoll, a high molecular weight polysaccharide, which exerts negligible osmotic pressure. The synaptosomes, as isolated with Ficoll, not having been exposed to hypertonic condition, are very sensitive to osmotic shock and are in general morphologically better preserved (Autilio, Appel, Pettis and Gambetti, 1968; Flexner, Gambetti, Flexner and Roberts, 1971; Cotman and Mathews, 1971; Morgan, Reith, Marinari, Breckenridge and Gombos, 1972). The purity of synaptosomes isolated with sucrose or with Ficoll will be discussed in a latter section.

Tamir, Rapport and Roizin (1974) have replaced sucrose with sodium diatrizoate. The main advantage of their method is in the quantity of synaptosomes obtained, the yield being twice that obtained with sucrose gradients while the purity of the preparations are comparable.

Bretz, Baggiolini, Hauser and Hodel (1974), using a continuous sucrose density gradient in a zonal rotor, reported the isolation of three distinct populations of synaptosomes

differing in apparent specific gravity, in biochemical properties and in the ability to selectively accumulate exogenous transmitters. The three synaptosome populations seemingly correspond to cholinergic, adrenergic and to GABA containing nerve endings.

Procedures using continuous sucrose gradients (Whittaker, 1968), zonal centrifugation (Cotman, Mahler and Anderson, 1968; McBride, Mahler, Moore and White, 1970; Spanner, 1972), and caesium chloride gradients (Kornguth, Anderson and Scott, 1969) have been described for the isolation of synaptosomes.

1.1.2 Isolation of Synaptic Plasma Membranes (SM)

Generally, synaptic membranes are prepared by lysing synaptosomes in hypotonic media although other methods have been used to disrupt the synaptosomes. The various components of lysed synaptosomes are then separated on sucrose density gradients.

Rodriguez De Lores Arnaiz, Alberici and De Robertis (1966) used the crude mitochondria for lysis and placed this lysate on a four step sucrose gradient in order to isolate SM. There seems to be some concern regarding the purity and origin of the membranes obtained (Jones, 1975).

A number of workers had observed that synaptosomes separated on Ficoll-sucrose gradients were purer and better preserved when compared to synaptosomes prepared on sucrose gradients (Autilio *et al.*, 1968; Flexner *et al.*, 1971). Cotman and Mathews (1971) took advantage of this by preparing synaptosomes in this way, lysing them by osmotic shock at alkaline pH, followed by separation on a discontinuous sucrose density gradient in order to obtain synaptic plasma membrane

of relatively high purity.

Gurd *et al.* (1974) and Morgan *et al.* (1971) reported the isolation of SM of higher purity than the membranes of Cotman and Mathews (1971). The major differences from the method of Cotman and Mathews are the use of PO_4-EDTA buffer and the washing of the crude mitochondrial fraction three times. Gurd *et al.* (1974) also used upward flotation of synaptosomes in Ficoll gradients.

The isolation of SM with very little mitochondrial contamination has been described (Bloom and Davis, 1970, 1973; Cotman and Taylor, 1972). The procedure involves lysing the crude mitochondrial fraction, reaction with succinate and iodonitroneotetrazolium violet and separation on a discontinuous sucrose gradient.

Jones and Matus (1974) report on the isolation of SM by lysis of the crude mitochondrial fraction followed by upward flotation in sucrose gradients.

A number of workers have also used continuous density gradients in zonal rotors (Cotman *et al.*, 1968; McBride *et al.*, 1970).

Levithan, Mushynski and Ramirez (1972) reported the isolation of SM by preparation of synaptosomes on discontinuous sucrose gradients followed by further separation on a continuous cesium chloride gradient with final separation of SM from lysed synaptosomes placed on a sucrose gradient.

1.1.3 Synaptic (Junctional) Complexes

De Robertis, Azcurra and Fiszer (1967), and Fiszer and De Robertis (1967) demonstrated that Triton X-100 will disrupt

the majority of the synaptosomal membrane leaving the contact or junctional region intact. A large proportion of the protein is solubilized along with (Na^+-K^+)-ATPase and AChE while the binding of certain cholinergic blocking agents remains unaffected. This indicated that the receptor components are present in the junctional region, as isolated after Triton X-100 treatment. They named this region the junctional complex which is defined as " . . . a unit composed of the two synaptic membranes, the cleft with the intersynaptic filaments and the subsynaptic web . . . ." (De Robertis *et al.*, 1967). Morphologically the complexes seem to consist mainly of postsynaptic membranes.

Cotman and Taylor (1972) treated a lysed crude mitochondrial fraction with succinate and iodonitroneotetrazolium violet. The synaptic plasma membranes can then be isolated relatively free of mitochondria since the treatment specifically increases the buoyant density of mitochondria. The synaptic plasma membranes so obtained are then treated with Triton X-100 from which synaptic complexes are isolated. Again, postsynaptic membranes seem to be a major component of the synaptic complex fraction. This is essentially the same method as reported by Davis and Bloom (1973). Both above reports stress the protective effect of calcium on the synaptic complexes.

Therien and Mushynski (1976) have isolated synaptic junctional complexes of high structural integrity. SM isolated according to the method of Bloom and Davis (1973)

were homogenized in 0.2% Triton X-100, 0.5% Dextran T-500, EDTA, and Freon TF-113 and then pelleted. Synaptic junctional complexes were then separated on sucrose density gradients containing 0.02% dextran sulphate. As much as 65% of the membrane material can be identified morphologically as junctional complexes with very little extrajunctional synaptic membrane.

1.1.4 Postsynaptic Membranes

Garey, Harper, Best and Goodman (1972) placed a synaptosome fraction, obtained from continuous Ficoll gradients, onto continuous sucrose gradients. One of the fractions obtained is composed mainly of postsynaptic membranes. The major contaminants are small unidentified membrane fragments.

Cotman, Banker, Churchill and Taylor (1974) have isolated postsynaptic densities (PSD) by treating SM with sodium lauroyl sarcosinate. The fraction is reported to contain 80% PSD.

A procedure which involves treating SM with Triton X-100 and sodium lauroyl sulfate has been reported for the isolation of PSD but the structures are disrupted and the purity was not established (Matus and Walters, 1975a; Walters and Matus, 1975).

A postsynaptic membrane fraction enriched in muscarinic and nicotinic receptors, and in 3',5'-nucleotide phosphodiesterase has been prepared by phospholipase A_2 treatment of synaptosomes followed by isolation on discontinuous sucrose gradients (Bartfai, Berg, Schultzberg and Heilbronn, 1976). The outside regions which face the cleft seem to

be preferentially conserved as opposed to Cotman's PSD which preserve mainly the inside region of the postsynaptic membrane.

Cohen, Blomberg, Berzins and Siekevitz (1977) were able to isolate postsynaptic membranes by treatment of synaptosomes or synaptic membranes with 1% Triton X-100 followed by separation on sucrose gradients.

1.1.5 Synaptic Vesicles

The term "synaptic vesicle" was introduced by De Robertis and Bennett in 1954. The isolation of these components is mainly the work of Whittaker and of De Robertis.

De Robertis *et al.* (1963) ruptured the synaptosomes present in the mitochondrial fraction by treatment with water and $CaCl_2$. The suspension is pelleted at 11,500g for 20 min to remove mitochondria, myelin, and ruptured synaptosomes. The supernatant is further pelleted at 100,000g for 30-60 min. This last pellet contains mainly synaptic vesicles.

Whittaker *et al.* (1964) similarly treated the mitochondrial fraction by osmotic lysis in water and then pelleted this suspension. The supernatant was placed on a discontinuous sucrose gradient, giving rise to seven fractions, one of which consists mainly of synaptic vesicles.

1.2 ASSESSMENT OF PURITY OF BRAIN SUBCELLULAR FRACTIONS

The use of markers is helpful for the analysis of various fractions obtained when a tissue is subjected to subcellular fractionation. Positive markers enable one to

monitor the purification or enrichment of the components of interest while the analysis for negative markers gives some indication as to the amount of contamination with other subcellular components. The results obtained from enzymatic or other chemical markers can then be compared to morphological data obtained from electron microscopic analysis. An overall assessment of purity of the fractions can then be made.

1.2.1 Enzyme Markers

1.2.1.1 Early work

Few markers that had been demonstrated to be localized in specific subcellular components of brain were available when subcellular fractionation of brain was first attempted. In general, markers that had been analysed and localized in liver subcellular fractions were assumed to have the same subcellular localization in brain, an assumption which is still valid for many markers.

Hebb and Whittaker (1958) monitored the crude purification of a synaptic vesicle containing fraction by assaying for acetylcholine (ACh), which was presumed to be associated with synaptic vesicles. Choline acetylase, which is involved in acetylcholine synthesis, was also measured. Both enzymes were found to have a similar distribution and both were located mainly in the synaptic vesicle containing subfraction of the crude mitochondria.

Whittaker (1959) demonstrated that most of the succinic dehydrogenase (SDH) activity could be found in subfraction C of the P_2 fraction which morphologically seemed to be composed

mainly of mitochondria. It was also shown that as much as 26% of the P_2 SDH activity could be found in subfraction B, the synaptic vesicle containing fraction. Acid phosphatase, a lysosomal marker, was localized mainly in the synaptic vesicle containing fraction as was 5-hydroxytryptamine, a putative neurotransmitter. ß-glucuronidase was found mainly in subfraction C while acetylcholinesterase (AChE) was associated principally with the microsomal fraction. Thus, Whittaker obtained a fraction enriched in synaptic vesicles that was extensively contaminated with mitochondria and lysosomes.

Gray and Whittaker (1960) and De Robertis *et al.* (1961b) reported that the synaptic vesicle containing fraction was composed mainly of pinched-off nerve endings which had resealed to form vesicles that were subsequently named 'synaptosomes' by Whittaker *et al.* (1964). Synaptic vesicles and mitochondria were shown to be contained within the synaptosomes.

De Robertis *et al.* (1962) isolated two fractions of nerve endings, one with high levels of AChE and ACh, the other with much lower levels of these two components. Thus cholinergic and non-cholinergic nerve endings had been partially separated. Rodriguez and De Robertis (1962) reported that as expected, monoamine oxidase was probably of mitochondria origin.

After these initial attempts at brain subcellular fractionation, other putative markers were investigated in

order to ascertain their usefulness in the identification of subcellular fractions. Albers, Rodriguez De Lores Arnaiz and De Robertis (1965) found (Na^+-K^+)-activated ATPase to be associated with cholinergic synaptosomes while K^+-activated p-nitrophenyl-phosphatase seemed to be concentrated in non-cholinergic synaptosomes. Glutamic acid decarboxylase was also found to be associated with non-cholinergic nerve endings (Salganicoff and De Robertis, 1963). This work was substantiated by De Robertis *et al.* (1966a) who reported that the AChE and (Na^+-K^+)-ATPase were enriched mainly in synaptic plasma membranes (SM) isolated from cholinergic synaptosomes while glutamic acid decarboxylase was found mainly in non-cholinergic SM. K^+-activated p-nitrophenyl-phosphatase could be demonstrated equally well in both types of SM.

1.2.1.2 Cytoplasmic markers

Lactate dehydrogenase has been used as a cytoplasmic marker and is also useful as an indicator of synaptosome intactness by measuring the occluded enzyme released after synaptosome lysis (Spanner, 1972). It is always important to measure at least one cytoplasmic marker in order to ensure that soluble proteins are not bound to membrane fractions.

1.2.1.3 Lysosomal markers

Acid phosphatase has been the most widely used lysosomal marker (Whittaker, 1959; Cotman and Mathews, 1971; McBride *et al.*, 1970). β-glucuronidase (Whittaker, 1959);

β-galactosidase (Morgan *et al.*, 1971), β-glucosidase (Morgan *et al.*, 1971; Van Leeuwen *et al.*, 1976) and β-N-acetylglucosaminidase (Cotman and Mathews, 1971; Bretz *et al.*, 1974) are also reported to be lysosomal markers.

1.2.1.4 Endoplasmic reticulum markers

RNA is a good marker for rough endoplasmic reticulum and has been useful in indicating that the crude mitochondrial fraction should be washed a few times in order to minimize microsomal contamination (Gurd *et al.*, 1974; Morgan *et al.*, 1971). It should be mentioned though that RNA is also present in the nuclear fraction and in mitochondria.

For smooth endoplasmic reticulum, the most widely used marker is NADPH : cytochrome c reductase (Gurd *et al.*, 1974; Morgan *et al.*, 1971; Van Leeuwen *et al.*, 1976). Glucose-6-phosphatase is a good microsomal marker in liver but its activity is very low in brain although it has been used for the analysis of microsomal contamination in brain subcellular membranes (Stephens and Sanborne, 1976; Van Leeuwen, *et al.*, 1976). PAPS : cerebroside sulfotransferase and UDP-glucose : ceramide glucosyl transferase have been reported as microsomal markers by Morgan, Breckenridge, Vincendon and Gombos (1973).

1.2.1.5 Mitochondrial markers

Monoamine oxidase and rotenone insensitive NADH : cytochrome c reductase have been the most widely accepted markers for the outer mitochondrial membrane (Gurd *et al.*, 1974; Morgan *et al.*, 1971; Van Leeuwen *et al.*, 1976) although the

rotenone insensitive NADH : cytochrome c reductase has been reported to be localized also in the endoplasmic reticulum (Beattie, 1968; Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967).

The two most appropriate markers for the inner mitochondrial membrane are cytochrome oxidase and succinate dehydrogenase (Cotman and Mathews, 1971; Gurd *et al.*, 1974; Morgan *et al.*, 1971). Rotenone sensitive NADH : cytochrome c reductase is also reported to be a mitochondrial marker (Van Leeuwen *et al.*, 1976).

The presence of mitochondrial matrix has been evaluated mainly be measuring malate deyhdrogenase (McBride *et al.*, 1970).

1.2.1.6 Plasma membrane markers

The most widely accepted plasma membrane markers are (Na^+-K^+)-activated ouabain sensitive ATPase, 5'-nucleotidase, and acetylcholinesterase (Bretz *et al.*, 1974; Cotman and Mathews, 1971; Gurd *et al.*, 1974; Hemminki and Suovaniemi, 1973; Morgan *et al.*, 1971).

Steck and Wallach (1970), on the basis of an extensive review of the literature have concluded that 5'-nucleotidase is the most specific and widespread plasma membrane activity known.

Jones and Matus (1974) state that " (Na^+-K^+)-ATPase is perhaps the best defined of all plasma membrane enzymes and the particular needs of the neuronal membrane to actively transport these ions, together with the shared sensitivity of this enzyme and the neuronal Na^+ pump to inhibition by

ouabain, commend its use as a neuronal plasma membrane marker."

AChE has been used extensively as a synaptic membrane marker but there is evidence that this enzyme is present in endoplasmic reticulum, on the surface of axons and in the axoplasm (Kokko, Mautner and Barrnett, 1960; Novikoff, 1967). Aldridge and Johnson (1959) report that the microsomal fraction has the largest amount of AChE as well as the highest specific activity.

Alkaline phosphatase (Cotman and Mathews, 1971; Stephens and Sandborn, 1976), K^+-stimulated phosphatase (Hemminki and Suovaniemi, 1973) and α-naphthylacetate esterase (Bretz *et al.*, 1974) are reported to be good plasma membrane markers for brain. Glutamate decarboxylase (Bretz *et al.*, 1974; Salganicoff *et al.*, 1963) and acetylcholine (Whittaker, 1972) are possibly more specific markers for nerve endings than the above mentioned plasma membrane markers but these two components are not markers for synaptic plasma membrane.

Gangliosides may possibly be good markers for synaptic membrane since synaptosome and SM fractions are reported to be particularly enriched with respect to these sphingoglycolipids (Morgan *et al.*, 1971; De Robertis, Lapetina and De Plazas, 1976; Eichberg, Whittaker and Dawson, 1964; Whittaker, 1966; Wiegandt, 1967). It should be mentioned, though, that high levels of gangliosides were also detected in the microsomal fraction, sometimes in an even larger

quantity than in SM (Seminario, Hren and Gomez, 1964; Spence and Wolfe, 1967). Gangliosides are reported to be also enriched in astrocyte and axon preparations (Poduslo and Norton, 1972; Dekirmenjian *et al.*, 1969).

1.2.1.7 Other markers.

One of the main markers for myelin is 2', 3', -cyclic nucleotide 3'-phosphohydrolase (Jones and Matus, 1974; Morgan *et al.*, 1971; Van Leeuwen *et al.*, 1976).

Measurements of cerebroside and of the encephalitogenic basic protein are also useful to indicate the presence of myelin. The Folch-Lees proteolipid protein and Wolfgram proteins, although being major components of myelin, have not been used to any extent for assessing myelin contamination in other subcellular fractions.

Catalase activity has been measured to indicate the presence of peroxisomes (Bretz *et al.*, 1974).

Fucose-^{3}H incorporation (Gurd *et al.*, 1974) and galactosyl transferase (Morgan *et al.*, 1973) activity indicate the presence of Golgi apparatus.

1.2.1.8 General comments concerning purity.

Cotman and Mathews (1971) reported that their method for the isolation of synaptic plasma membrane provided membranes of acceptable purity with a yield of 1.5-2.0 mg protein per gm brain (wet weight). There was very little contamination with mitochondrial membranes since the SM contained only 7% of the total synaptosome cytochrome oxidase but there was significant contamination with

lysosomes since the SM contained 44% of the total synaptosome lysosomal activity. Myelin and microsomal contamination was not studied. (Na^+-K^+)-ATPase was enriched five fold with most of the total activity being found in the SM Fraction 2. The SM fraction also contained increased activities of 5'-nucleotidase and AChE but these two enzymes were detected primarily in subsynaptosomal Fraction 1.

Morgan *et al.* (1971), with a method quite similar to that of Cotman and Mathews (1971), recorded that the purified SM obtained was essentially free from contamination with inner mitochondrial membranes, rough endoplasmic reticulum, lysosomes, myelin and soluble enzymes. The contamination with outer mitochondrial membrane and smooth endoplasmic reticulum was calculated to be about 10 and 5% respectively, yielding a preparation containing 85-90% of plasma membrane. The (Na^+-K^+)-ATPase activity was enriched more than ten fold.

Gurd *et al.* (1974) demonstrated that their SM fraction was contaminated with approximately 15% of microsomes and 10% of mitochondria. The (Na^+-K^+)-ATPase was enriched by ten fold.

The three procedures just mentioned used Ficoll gradients for the preparation of synaptosomes. The advantage of Ficoll gradients is that the synaptosome fraction is contaminated to a lesser degree with mitochondria and myelin (Appel *et al.*, 1972; Cotman, 1972) and with glial cells (Cotman *et al.*, 1971a) as opposed to synaptosomal fractions separated on sucrose gradients. Of the three reports mentioned

above, that of Gurd *et al.* (1974) and of Morgan *et al.* (1971) washed the crude mitochondrial fraction extensively, thereby eliminating much of the microsomal contamination.

Since many of the contaminants (microsomes, glial surface membranes and outer mitochondrial membranes) exhibit densities in sucrose overlapping that of SM, the use of Ficoll gradients and washing of the crude mitochondrial fraction underline the importance of purifying the synaptosomes before they are lysed in order to obtain SM of the highest purity.

Cotman and Mathews (1971) calculated that 10-20% of particulate material in brain homogenate is neuronal plasma membrane. Therefore an enrichment of only 5-10 fold is to be expected for plasma membrane markers. Morgan *et al.* (1971) calculate that an enrichment of approximately 12 fold can be expected for neuronal plasma membrane.

One of the major problems encountered in determining the enrichment of SM is that most of the positive markers are not specific for synaptic membranes but are expected to also be present on neuronal and glial plasma membrane, on axons, and on dendrites (Barondes, 1974). Thus, acetylcholinesterase has been demonstrated histochemically to be present on axonal and dendritic membranes, and on endoplasmic reticulum (Kokko, 1969; Novikoff, 1967). Also, 5'-nucleotidase has been detected in glial cells by histochemical methods (Torrack and Barrnett, 1964). Cotman and Mathews (1971) suggest that 5'-nucleotidase is possibly present in

higher concentration in axonal, dendritic or glial membranes than in synaptic membranes.

One of the few reports to study glial contamination using chemical markers is that of Morgan *et al.* (1972) who concluded that the SM could contain 5-10% of glial membranes.

Levithan *et al.* (1972) added radioactive myelin, glia, axons, mitochondria, and endoplasmic reticulum to a brain homogenate before isolation of SM and calculated the total contamination of the SM, with the labelled membranes and cells, to be in the order of 10-15%. This correlated quite closely with calculations arrived at when enzyme markers were used.

In the assessment of purity or contamination of any fraction, it is probably important to consider De Duve's (1963) conclusion, as summarized by Whittaker (1965), " . . . . unless a fraction is completely homogeneous it is usually impossible to be certain whether a particular activity is to be associated with the major component or with a minor component of high specific activity."

1.2.2 Morphological Assessment.

Whittaker's (1965) description of synaptosomes was described in an earlier section (see Isolation of Synaptosomes). A more detailed recent description is given by Jones (1975) " . . . . the majority of cortical synaptosomes have the following features : (a) a presynaptic network in which a hexagonal arrangement of the constituent strands

can sometimes be identified, (b) trilaminar pre- and post-synaptic membranes, (c) dense projections on the presynaptic side of the junction, (d) cleft densities within the cleft, and (e) postsynaptic focal densities on the postsynaptic side of the junction and corresponding in general position to the adjacent cleft densities." The presynaptic portion is enclosed by a continuous membrane the diameter of this synaptosome being in the range of 0.5-0.6 μm. Synaptic vesicles and intraterminal mitochondria are also present within the enclosed vesicle. Some synaptosomes have transverse bars in place of cleft densities (Jones, 1975).

The detailed morphological analysis of synaptosomes was possible mainly due to the development of the techniques of glutaraldehyde fixation followed by phosphotungstic acid (PTA) staining (Bloom and Aghajanian, 1966) and of substituting a bismuth iodide complex for PTA and then staining with uranyl acetate and lead hydroxide (Pfenninger, Sandri, Akert and Eugster, 1969). Both of these methods highlight the paramembranous densities.

The assessment of the purity of synaptosomal preparations basically involves the positive identification of synaptosomes with some or all of the features described above and a semi-quantitative calculation of contaminants which are present such as: free mitochondria, myelin, and endoplasmic reticulum.

Analysis of synaptosome preparations has led to the conclusion that some of the contaminants are mitochondria,

membrane vesicles some of which contain ribosomes, free membranes, neurofilaments, and myelin (Cotman and Mathews, 1971). The authors mention that approximately 60-75% of the components are synaptosomes with free mitochondria accounting for another 10%. Stephens and Sandborne (1976) report that endoplasmic reticulum can be seen attached to the free mitochondria.

One of the major problems encountered in morphological analyses of synaptosome preparations is that due to differences in the sedimentation coefficients of particles, layering occurs in the pellets. Electron micrographs may therefore not be representative of the preparation unless precautions are taken such as sectioning pellets at different depths and in different planes (Jones, 1975).

Lemkey-Johnston and Dekirmenjian (1970) reported that the synaptosome fraction, as prepared by the method of Whittaker, contain approximately 48% axons, 25% synaptosomes, 2% mitochondria and 22% membrane of undetermined origin, as calculated by membrane surface measurements. Microsomes were also present in the preparations. Due to a number of changes in the isolation procedure, such as the use of whole brain, Jones (1975) suggests that the heterogeneity of routine synaptosomal preparations was not proved by these studies but the possibility of axonal contamination is now recognized.

Electron microscopic analyses of synaptosomal fractions has led to another important consideration, namely that the

pinched off nerve endings may contain endogenous endoplasmic reticulum. The tubules of smooth endoplasmic reticulum have been found along the entire length of the axon and in the nerve ending where they divide into a primary network which gives rise to a secondary network of thin canaliculi (Droz, 1975; Droz, Rambourg and Koenig, 1975). Synaptic vesicles can be seen budding from the canaliculi (Droz, 1975; Pappas, 1975) while other canaliculi approach the presynaptic membrane (Droz, 1975).

The analysis of purity of SM involves the assessment of purity of synaptosomes from which the SM were isolated, the positive identification of pre- and post-synaptic densities and an evaluation of identifiable contaminants although the majority of membranes present are not identifiable.

1.3 BRAIN ANTIGENS

The immunological approach has been used successfully for the study of the membrane structure of a number of cells such as lymphocytes, erythrocytes and tumor cells and has recently been applied to the study of the brain.

Immunochemical methods, being precise, sensitive, and specific, provide markers for glial cells and neurons and possibly to differentiate between various types of neuronal cells. In addition it may be possible to distinguish the various neuronal subcellular membranes and to gain some information as to the molecular organization of such

membranes.

Attention has been focused mainly on the synaptic plasma membrane (SM) since: (1) there is a reasonable chance of finding specific antigens in this specialized membrane, (2) information on the molecular structure of SM would aid in the understanding of synaptogenesis, and (3) the use of antibodies *in vivo* would provide a functional dissection of components involved in learning, memory and synaptic transmission.

This section is therefore a brief review of the antigenic components of the brain with special emphasis on tissue specific soluble components and membrane-bound antigens.

1.3.1 Soluble Antigens

Of the 100 or more water soluble proteins of brain (Moore and McGregor, 1965; Bogoch, Rajam and Belval, 1964) only 12 from rat brain were found to stimulate the production of detectable precipitating antibodies in rabbits (MacPherson and Liakopoulou, 1966). Only one of the antigens was found to be organ specific. One was detected only in brain, spinal cord and peripheral nerve and has been refered to as a "species-restricted antigen of nervous tissue" (SRANT) (Liakopoulou and MacPherson, 1970). SRANT was demonstrated to exist in two molecular forms, each with a molecular weight of 70,000. Similarly, 17 immunogens could be detected in saline extracts of bovine brain, only five of which were organ specific (Hatcher and MacPherson, 1969). One of the

immunogens has a molecular weight of 84,000, the electrophoretic mobility of an α globulin and contains 2% carbohydrate (Hatcher and MacPherson, 1970). This water soluble bovine antigen specific for nervous tissue (α-BASNT) was different from other identified brain proteins.

Similarly, Kosinski and Grabar (1967) detected only 11 antigenic components in water extracts of rat brain, five of which were brain specific.

By the use of antigen-antibody crossed electrophoresis, Bock, Mellerup and Rafaelsen (1971) could detect up to 27 rat brain antigens. Only five were brain-specific, one being also species restricted (Bock, 1972).

Apart from SRANT and α BASNT which have already been mentioned, a number of other organ specific antigenic components of the brain soluble fraction have been isolated. Thus S-100 protein was first reported by Moore (1965) and has since been extensively studied. The molecular weight of this cytoplasmic protein has been reported to be in the order of 21-24,000 daltons but seemed to be composed of three subunits of 7,000 daltons (Moore, 1975a). The glial origin of S-100 has been confirmed by McEwen and Hyden (1966) and the controversy as to the homogeneity of this protein has been partially resolved by Hyden and McEwen (1966) and by Callissano (1973) who presented evidence for the formation of multiple forms in the presence of calcium. Still unresolved is whether S-100 as isolated by Moore (1965) is homogeneous or whether it exists as a family of very acidic proteins, with similar size but differing in composition as

suggested by Mahadik, Korenovsky, Graf and Rapport (1977).

Another acidic protein, 14-3-2, was detected only in neurons (Moore, 1965). The neuronal origin of this protein has been confirmed by immunohistochemical methods by Grasso, Haglid, Hansson, Persson and Rönnbäck (1977) and by Rönnbäck *et al.* (1977) who detected 14-3-2 in cytoplasm, on plasma membranes, endoplasmic reticulum, and on the nuclear membrane.

A nervous system protein (NSP-R) from rat brain has been reported to be antigenically similar to the bovine 14-3-2 but to differ in molecular weight and amino acid composition (Pickel, Reis, Marangos and Zomzely-Neurath, 1976; Zomzely-Neurath, Marangos and Keller, 1977). There is evidence that NSP-R represents a brain specific form of enolase.

Bennett and Edelman (1968) isolated an acidic protein (antigen α), with a molecular weight of 39,000, from the soluble fraction of peripheral and central nervous tissue.

A water soluble brain specific glycoprotein, probably of glial origin and which migrates elctrophoretically in the α_2 region at pH 8.2, has been isolated and characterized (Warecka and Bauer, 1967; Warecka, 1972).

Other brain or tissue specific soluble antigens have been reported: the glycoprotein GP-350 (Van Nieuw Amerongen, Van den Eijnden, Heijlman and Roukema, 1972), the 10B and 11A glycoproteins (Bogoch, 1970), the glial fibrillary acidic protein (Eng, Vanderhaeghen, Bignami and Gerstl, 1971), the glycoprotein, GM 50-c (Ramirez, Haglid, Karlsson and

Rönnbäck, 1974), and a β_1-β_2 globulin (Orosz, Falus, Madarasz, Gergely and Adam, 1974a).

1.3.2 Membrane Bound Antigens

With the advent of subcellular fractionation techniques capable of resolving individual membrane systems in relatively pure form, immunological methods have been increasingly used for the study of membrane-bound antigens of brain.

It was expected that the use of synaptosomes for the production of antisera in contrast to the use of whole brain or individual brain areas would lend some refinement to the search for synaptic membrane specific antigens. Thus De Robertis *et al.* (1966b and 1968) were able to demonstrate antibodies that reacted with synaptosomes in complement fixation tests, formed precipitin bands in immuno-diffusion plates, and produced complement dependent lysis of nerve endings. The antisynaptosomal sera also reacted with the myelin, mitochondrial and microsomal fractions. Herschman *et al.* (1972) reported on an anti-synaptosome serum which did not significantly fix complement in the presence of nuclei, myelin, synaptic vesicles or soluble fraction of brain but which did react with mitochondria and synaptosomes. The antigens studied seemed to be brain specific since the antiserum reacted only very weakly with subcellular fractions of other organs.

Since synaptosomes are not very pure and since they contain mitochondria, synaptoplasm, synaptic vesicles and endoplasmic reticulum, it is not surprising that anti-

synaptosome sera would contain antibodies reactive with several subcellular components. Therefore, a number of studies have addressed the problem of specificity and cross-reactivity of anti-synaptic membrane sera.

Mickey *et al.* (1971), using the technique of sequential adsorption of an ^{125}I-labelled anti-SM serum, were able to demonstrate that SM shared antigenic components with brain mitochondria and myelin. After absorption of the anti-serum with myelin, brain mitochondria and synaptosomes only 6% of the original activity seemed to be specific for SM. Similarly, an anti-brain mitochondrial serum reacted with liver mitochondria and synaptosomes while only 11% of the activity was specific for brain mitochondria.

Raiteri *et al.* (1972) and Raiteri and Bertollini (1974) studied anti-synaptosome sera as opposed to anti-SM sera but the nature of their assay is such that the only antibodies measured would be those directed against antigenic determinants located on the external surface of synaptosomes. The quantitative assay involves the measurement of the decrease of synaptosomal swelling in glycerol solutions after incubation with antibody and complement. The results indicated that synaptosomes share some antigenic components with myelin and others with mitochondria. Some components are shared by myelin, mitochondria and synaptosomes. After extensive absorption with myelin, 40% of the original antibody activity was demonstrated to be specific for synaptosomes. Raiteri *et al.* (1973) reported that no correlation exists between

complement fixation tests and the swelling test as some anti-SM and anti-synaptosome sera with high complement fixation activity had low swelling inhibition activity and vice versa.

Lim and Hsu (1971) measured the activity of an anti-SM serum by the complement fixation test and concluded that the highest complement fixation titer was obtained with SM followed closely by microsomes. Synaptic vesicles, myelin and homogenates from other organs also gave significant complement fixation titers.

MacPherson *et al.* (1973) demonstrated by immunodiffusion analyses that some antigens are shared by SM and microsomes.

Stallcup and Cohn (1976), studied the surface antigens of cloned glial and neuronal cell lines and concluded that there are antigenic components specific for glia and for neurons and that some are shared by both cell types. Similarly, Mihailovic and Hydén (1969) were able to demonstrate the specificity of anti-glia and anti-neuron antisera as well as the sharing of some antigenic components.

In 1974, Jorgensen and Bock, using the technique of crossed immunoelectrophoresis demonstrated five brain specific antigens in Triton X-100 extracts of SM. The antigens were labelled D1, D2, D3, D5 and D12. Bock and Jorgensen (1975) reported that synaptic vesicles antigen C1 is also present in SM while antigens D1, D2 and D3 are found in SM but not synaptic vesicles. Bock *et al.* (1975) used the term synaptin for antigen C1.

Bock and Hamberger (1976) demonstrated that significant quantities of C1, D1, D2 and D3 could also be detected in glial extracts. The presence of these neuronal antigens in glia could probably be explained in terms of SM or synaptic vesicle contamination of the glial preparations since Jacque *et al.* (1976) reported that levels of synaptin, D1, D2, and D3 were not quantitatively changed in Jimpy mice. In these myelin deficient mutants, there is a large decrease in the total number of glial cells and if the SM antigens are components of glial cells, a measurable decrease in the levels of these antigens should be expected.

Using a phospholipid-free homogenate of cat cerebral cortex as immunogen, an antiserum was obtained which, after absorption with serum, liver and kidney extracts, and brain soluble fraction, still formed one precipitin line when tested with Triton X-100 extracts of SM. No reaction with synaptic vesicles, mitochondria, microsomes or myelin could be observed (Orosz, Hamori, Falus, Madarasz, Lakos and Adam, 1973; Orosz, 1975). The antigen seems to be a glycoprotein with a molecular weight of 40-50,000 daltons (Orosz, Madarasz, Falus and Adam, 1974b). F(ab')2 fragments were prepared, isolated, and conjugated with ferritin. The label was observed, to be specifically bound to the synaptosome fraction, on the post-synaptic region or sub-synaptic web. No label could be detected in the synaptic cleft, on presynaptic membranes or in the cytoplasm. Fluorescent antibody and ferritin-labelled antibody were used to localize the antigen in cultured

Purkinje neurons from rats of various ages. In cultures from five-day old rats, the antigen could be detected only on the surface of nerve cells, always on those parts of the plasma membrane which were at or near to connections with other cells. The antigen was localized mainly in the post-synaptic region and only partly on the plasma membrane of neurons from ten-day old rats. In adult rats the antigen was detected only in the postsynaptic densities (Orosz *et al.*, 1973). The authors suggest that the antigen detected in young rat neurons is a precursor of an adult postsynaptic antigen and have called this a nerve cell-specific "differentiation" antigen.

That S-100 seemed to be mainly of glial cytoplasmic origin was discussed in an earlier section. Haglid, Hansson and Rönnbäck (1977) demonstrated by immunofluorescence and immunoelectron microscopy that anti S-100 antibodies localized in glial cells as well as in neurons and could be detected on the neuronal plasma membrane, the nuclear membrane, and especially in the post-synaptic membrane.

Evidence was presented that synaptosomes contain 8% of the total cortical S-100 and that 15% of the synaptosomal S-100 appears to be membrane bound as it is released only by pentanol, Triton X-100 or SDS (Donato, Michetti and Miani , 1975; Donato, 1976).

Mahadik, Korenovsky, Graf and Rapport (1977) have indicated that there are a number of acidic proteins that migrate as one band in 12% polyacrylamide gels and react

with anti S-100 serum, but differ in their subunit composition. Thus the existence of this "family" of S-100 proteins may account for the ubiquitous localization of S-100.

Biochemical and immunohistochemical analyses have indicated that tubulin is a prominent protein of synaptic complexes. In brain, anti-tubulin antibodies reacted only with microtubules and with antigens localized on the cytoplasmic side of the postsynaptic membrane (Matus, Walters and Mughal, 1975; Walters and Matus, 1975b).

Rönnbäck, Persson, Hansson, Haglid and Grasso (1977) reported that anti 14-3-2 antibodies labelled the neuronal plasma membrane, including the axon, dendrites, post- and pre-synaptic membranes, nuclear membrane and the endoplasmic reticulum cisterns.

Van Nieuw Amerongen and Roukema (1974) were able to demonstrate that a significant proportion of GP-350 is membrane-bound and that most of this activity could be recovered in SM fractions. Similarly, preliminary work has indicated that GM 50-C is probably associated with SM (Ramirez *et al.*1974).

1.3.3 Histochemistry

Immunohistochemical techniques have been very useful for the localization of antigenic components especially when mono-specific antisera are available but these techniques have yielded useful information when applied to complex antigen-antibody systems as well.

Thus, Kornguth *et al.* (1969) reported that fluorescent

anti-SM antibodies localized mainly on the plasmalemmae of granule cells and Purkinje cell bodies and dendrites. Fluorescent areas near the Purkinje cell dendrites appeared to be discrete particles and thus may have been synaptic contacts. In the cerebral cortex, fluorescence was seen mainly in the region of the neuronal soma. Matus, Jones and Mughal (1976) found that fluorescein-labelled anti-SM antibodies stained all regions of the neuropile intensely. White matter, cytoplasm and non-nervous tissue did not stain and adsorption of the anti-serum with mitochondria or myelin had no effect on the staining pattern. In the cerebellar mossy fibre endings, staining was limited to the terminal region; axons, perikarya, and dendrites did not stain.

By immunofluorescent histochemistry of the chick forebrain, anti-SM serum was found to stain structures approximately 3 μm in diameter, structures which could represent synaptic terminals (Rostas and Jeffrey, 1973; Livett, Rostas, Jeffrey and Austin, 1974). Throughout the brain, only areas rich in axons and nerve endings were stained while cell bodies were not stained. The antigen could not be detected in glia, perikaryal membranes, and perikaryal cytoplasm (Rostas and Jeffrey, 1975). Only the presynaptic and axolemmal membranes were stained. The authors suggested that there is possibly some barrier to lateral movement of membrane components between the perikaryal and axonal membranes. Evidence was presented that, after ligation of the sciatic nerve, the antigens accumulated on the proximal side of the ligation

while no fluorescence could be detected on the proximal side, indicating axonal transport of the antigens (Rostas and Jeffrey, 1977). It is therefore surprising that the antigens could not be detected in axoplasm of nerves which had not been ligated. The axolemmal staining that had been reported before ligation may in effect have been axoplasmic staining since antigens could not be detected on the distal side of ligations.

It is noteworthy that such discrete cellular and sub-cellular distribution of antigens could be demonstrated since the antiserum was not monospecific as four antigen-antibody systems could be detected by immunodiffusion tests (Rostas and Jeffrey, 1975). The synaptic membrane used as immunogen was contaminated with approximately 15 and 25-30% of mitochondrial and microsomal membranes respectively (Howe, Fenwick, Rostas and Livett, 1977). Although the antiserum had been absorbed with liver membranes, one would expect anti-brain microsome and anti-brain mitochondria antibodies to be present in the serum. Since the antiserum was diluted 1:100 (Howe *et al.*, 1977), it is possible that antibodies, specific for the contaminants, would not have been detected.

1.3.4 *In Vivo* Studies

1.3.4.1 Effects on electrical activity of brain

Increased attention to the immunological approach for the study of brain function was mainly due to the report of Mihailovic and Jankovic (1961) who demonstrated that intra-ventricular injection of antisera raised against the soluble fraction of cat caudate nucleus induced a modification of

electrical activity of the caudate nucleus. Other areas of the brain were relatively unaffected and anti-cat hippocampus serum had no effect on the caudate nucleus. No immunochemical evidence was presented that anti-brain antibodies were present or that demarcated areas of the brain contain specific antigens. Such antibody effects on specific areas of the brain has not been confirmed by other workers.

Antiserum raised against the ventral nerve cord of lobsters blocked the normal electrical membrane activity when the serum was applied onto the exposed giant axons of lobsters (Mihailovic, Jankovic, Beleslin, Milosevic and Cupic, 1965). Changes in the electrical activity were observed when anti-lobster brain serum was applied to the surface of cockroach brain (Jankovic, Rakic and Sestovic, 1969).

De Robertis *et al.* (1966b) demonstrated that an anti-synaptosome serum produced epileptiform spike-dome discharges in electrocorticograms when applied to the visual cortex of cat. Wald, Mazzuchelli, Lapetina and De Robertis (1968) reported that when the anti-cat synaptosome serum was applied to land snail neurons, a complement dependent progressive deterioration of bioelectrical activity occurred.

Jarosch and Precht (1972) reported that the trans-synaptic component of the parallel fiber-evoked field potential was strongly suppressed when anti-synaptosome serum was placed on the exposed surface of the cerebellar cortex. The antiserum effect was reversible and not complement dependent.

Recurrent epileptiform activity, recorded by the electroencephalogram (EEG), resulted when anti-SM serum was injected intraventricularly into rats and rabbits (Karpiak, Bowen and Rapport, 1973; Karpiak, Rapport and Bowen, 1974; Karpiak, Serokosz and Rapport, 1976). The abnormal electrical discharges lasted for as long as eight days and neither anti-S-100 nor anti-myelin sera caused the effect. Evidence was presented that anti-ganglioside sera also caused epileptiform spiking in the EEG (Karpiak, Graf, and Rapport, 1976).

Antisera to a synaptosomal actomyosin-like protein also induced focal epileptogenic activity when applied to rat brain (Bowen, Kosarova, Casella, Nicklas and Berl, 1976).

Such reports on the alteration of brain electrical activity supports the hypothesis that an autoimmune process may be involved in epilepsy (Karpiak *et al.*, 1974).

1.3.4.2 Effects on learning and memory

Jankovic, Rakic, Veskov and Horvat (1968) obtained significant changes in conditioned responses of cats after the animals had been injected intraventricularly with antisera raised against delipidated saline extracts of cat brain.

MacPherson and Shek (1970) found that rats immunized with rat brain or liver microsomes required a significantly greater number of trials to learn a visual discrimination problem than rats injected with saline. Thus, species-specific antigens seemed to be as important as brain-specific antigens with respect to their localization in

the brain cells responsible for learning. These results were confirmed and extended in that intraventricular administration of rat anti-rat brain microsome IgG or anti-liver microsome IgG produced the same effects. Control animals injected with normal rat IgG had no such impairment of learning (MacPherson and Chinerman, 1971). Using passive haemagglutination and complement fixation tests, the antigens were demonstrated to be membrane bound and to be present in both rat liver and brain microsomes (Shek and MacPherson, 1971).

Intraventricular injection of anti-SM serum induced significant behavioral alterations of two caudate-mediated tasks (Karpiak *et al.*, 1974). This work was extended by Karpiak and Rapport (1975) who took advantage of the absence of a blood-brain barrier in fetal rats. Pregnant females were injected intravenously with anti-SM sera and, two months after birth male offspring showed significant behavioral deficits on a conditioned behavior. No behavioral changes could be detected in rats that had been exposed to anti-galactocerebroside serum. Rats receiving topical cortical injections of anti-SM or anti S-100 protein had impaired maze learning ability as compared to controls or to rats that received anti-myelin serum (Karpiak, Serokosz and Rapport, 1976).

Anti-brain ganglioside serum was found to be effective in inhibiting passive avoidance learning in rats (Karpiak, Graf and Rapport, 1976; Rapport and Karpiak, 1977). Evidence

was presented that anti-ganglioside serum inhibits the consolidation phase of learning and not the acquisition phase (Karpiak, Sowin and Rapport, 1977).

The effect of intraventricular injection of anti-SM IgG on recall of four different learning experiences was studied by Kobiler, Fuchs and Samuel (1976). Recall of the test animals varied for the four experiments but in each case there was a marked decrease in memory. In one experiment, partial recovery was detected (48 hours after antibody injection), indicating that the brain damage was probably not permanent.

Such results point to the usefulness of the immunological approach for "dissecting" the parameters of learning and memory.

1.3.4.3 Other *in vivo* effects

An antiserum specific for the isolated acetylcholine receptor was reported to produce paralysis in rabbits (Karlin, 1975). This result was confirmed by Clementi, Conti-Tronconi, Berti, and Folco (1976) who found that the paralysis caused by anti-cholinergic receptor serum could be inhibited by the administration of eserine. Such studies demonstrate that this experimental autoimmune disease may be a valid model for the study of human myasthenia gravis.

1.4 BIOCHEMICAL ANALYSIS OF SM, SJC, AND PSD

Many reports had demonstrated the resolution of SM proteins in polyacrylamide gels, starch gels or column

chromatography but Banker, Crain and Cotman (1972) were the first to report molecular weights for the individual polypeptide components. Electrophoresis of SM components on polyacrylamide gels (PAGE) in the presence of SDS and a reducing agent resolved 30 polypeptides with the bands corresponging to molecular weights of 99,000, 52,400 and 41,500 representing 40-50% of the total stained protein. Three of the bands seemed to be glycoproteins. Mitochondria and myelin treated in the same way gave different patterns.

These results have been confirmed by numerous reports. Quantitative and minor qualitative differences from the results of Banker *et al.* (1972) have been obtained, depending on the method used for SM isolation and depending on the system used for resolution of the polypeptides, but the overall pattern remains the same. Thus Wannamaker and Kornguth (1973) reported the major components to have molecular weights of 92,000, 53,000, 43,000, and 36,000. Similarly, Gurd *et al.* (1974) found that the major bands have molecular weights of 93,000 and 53,000 and that 6 or 7 glycoproteins could be detected.

The two predominant components of SM were suggested to have molecular weights of 92,000 and 50,000 (Jones, Mahler and Moore, 1975), while 6 to 9 glycoproteins could be demonstrated (Mahler, Gurd and Wang, 1975). Eleven of the bands obtained account for 95% of the total protein (Wang and Mahler, 1976).

Cotman, using different SM preparations and different

resolution methods from those used in 1972 (Banker *et al.*) have now reported that the major polypeptides have molecular weights of 85,000, 55,000, 52,000 and 45,000 (Kelly and Cotman, 1977a).

There seems to be general agreement that the patterns obtained with SM, by PAGE in the presence of SDS, are different from the profiles obtained using myelin or mitochondria although there are a number of bands which seem to be the same for SM and mitochondria. This is not the case with microsomes in that there seems to be considerable similarity and overlap, both qualitatively and quantitatively, between the protein constituents of SM and the microsomal fraction (Jones *et al.*, 1975; Wannamaker and Kornguth, 1973; Gurd *et al.*, 1974; Mahler *et al.*, 1975). In this respect, Gurd *et al.* (1974) suggest that since separation of proteins in SDS is dependent on molecular weight, the possibility of minor compositional differences is still present. Barondes (1974) proposes that specific synaptic membrane components cannot be excluded considering the fact that synaptosomes and neuronal plasma membranes are significant components of the "microsomal fraction".

A number of components present in SM have been tentatively identified. Thus the localization of tubulin on the cytoplasmic side of postsynaptic membranes has been demonstrated by immunohistochemical methods (Matus *et al.*, 1975). Tubulin, the native protein of brain microtubules, has a molecular weight reported as 110,000 daltons, with two sub-

units of 55,000 daltons each (Shelanski, 1973). A component, with a molecular weight of 54,000 and accounting for 21% of the total membrane protein, comigrated with brain tubulin on SDS polyacrylamide gels (Mahler *et al.*, 1975; Wang and Mahler, 1976). Similarly, another component was tentatively identified as actin or actin-like protein. This protein with a molecular weight of 44,000 contributed 11% of the total SM protein. The possibility that bands found in the region of 200,000 to 250,000 daltons may be contributed in part by myosin-like proteins has been suggested (Mahler *et al.*, 1975) but there seems to be some doubt as to the validity of this assessment (Wang and Mahler, 1976). That actomyosin-like (neurostenin), actin-like (neurin) and myosin-like (stenin) proteins can be isolated from synaptic membrane has been confirmed by Berl (1975a and b) although the myosin-like protein seems to be associated mainly with synaptic vesicles. Neurostenin is reported to contribute 8-10% and 1-2% of bovine and rat synaptosomes respectively.

Components with molecular weights of 37,000 and 24,000 daltons comigrate with troponin T and I respectively (Wang and Mahler, 1976) although Berl (1975b) could not demonstrate troponin-like protein in synaptosomes.

Since various enzymes are used as SM markers, one would expect that some of the bands obtained in polyacrylamide gel electropherograms could be identified as enzymes. Thus Wenthold and Mahler (1974) isolated 6 isoenzyme forms of AChE, all with the same basic glycoprotein subunit of 80,000

daltons. (Na^+-K^+)-ATPase was presumed to be responsible for a component of 90,000 daltons (Mahler *et al.*, 1975; Wang and Mahler, 1976).

In this review, components of synaptic junctional complex and the post-synaptic density are considered separately from SM; the main reason being that there are major differences in polyacrylamide gel electropherograms obtained with SM, SJC and PSD. Since plasma membrane, adjoining the synaptic thickenings, contribute a large proportion of the total protein of SM, quantitative differences are to be expected when compared to SJC or PSD.

That treatment of SM with Triton X-100 releases 35-40% of the total protein, leaving intact synaptic junctional complexes, has been well documented (Fiszer and De Robertis, 1976; Cotman, Levy, Banker and Taylor, 1971b; Cotman and Taylor, 1972; Levitan, Mushynski and Ramirez, 1972; Davis and Bloom, 1973) although up to 60% of the protein can be solubilized under certain conditions (Cotman and Taylor, 1972; Davis and Bloom, 1973).

The SJC is reported to consist of 80% protein and approximately 20-25% lipid, half of which is phospholipid. Amino acid analysis of SJC gave values similar to those of SM (Churchill, Cotman, Banker, Kelly and Shannon, 1976).

Hydrophobic interactions may be important for the structural integrity of SJC since these structures were reported to be resistant to high salt concentrations, urea, high pH or moderate concentration of detergent. The structures, though, are sensitive to attack by various

proteolytic enzymes (Cotman and Taylor, 1972; Cotman, 1976).

Polyacrylamide gel patterns of SJC were reported as being very similar to SM profiles but with quantitative differences (Kelly and Cotman, 1977a).

Up to 30 components were detected in SJC with proteins of molecular weights corresponding to 42,000 and 53,000 daltons accounting for a large part of the total protein (Levitan *et al.*, 1972). Therien and Mushynski (1976) demonstrated that proteins of 55,000 and 45,000 daltons comigrate with tubulin and actin respectively. That β and γ actin isoproteins are integral components of SJC has also been reported by Kelly and Cotman (1977 b and c).

Synaptic junctional complexes contained the same amount of protein-bound carbohydrate as did SM while lipid-bound carbohydrate was not detected (Churchill *et al.*, 1976).

Gurd (1977) could detect only two Con-A receptors with molecular weights of 105,000 and 130,000 daltons in polyacrylamide electrophoretograms of SJC while Kelly and Cotman (1977a) were able to detect four such components with molecular weights of approximately 162,000, 123,000, 113,000 and 97,000 daltons.

The postsynaptic density has been increasingly studied during the last few years. This structure is composed of 10% lipid, 3% carbohydrate with the remainder being protein (Banker, Churchill and Cotman, 1974).

That tubulin is present in PSD's has been indicated using immunohistochemical techniques (Matus *et al.*, 1975;

Walters and Matus, 1975b). A component with a molecular weight of 55,000 daltons, which accounts for 14% of PSD protein, comigrates with tubulin on polyacrylamide gels (Kelly and Cotman, 1977a and b). Peptide mapping (Kelly and Cotman, 1977a; Walters and Matus, 1975b) and immunological studies (Kelly and Cotman, 1977a) have provided strong evidence that tubulin is in effect a major component of PSD's.

That the β- and γ-actin isoproteins are also present in PSD's has been demonstrated by comigration in polyacrylamide gels, peptide mapping, immunological assays, and amino acid analysis (Kelly and Cotman, 1977a, b, and c; Therien and Mushynski, 1976; Blomberg, Cohen and Siekevitz, 1977). This protein, with a molecular weight of 45,000 daltons, accounted for 6% of the PSD protein (Kelly and Cotman, 1977a; Blomberg *et al.*, 1977).

Blomberg *et al.* (1977) reported that a protein, with a molecular weight of 18,000 daltons may be troponin C.

A band with a molecular weight corresponding to 50-53,000 daltons was demonstrated by PAGE to be, by far, the major component of PSD's and to account for 45-50% of the total PSD protein (Kelly and Cotman, 1977a and b; Therien and Mushynski, 1976; Cohen *et al.*, 1977). The available evidence indicated that this protein could account for 70% of the total polypeptide chains present in PSD's (Banker *et al.*, 1974). A number of reports had suggested that this was tubulin or a tubulin-like protein but evidence from

immunological studies, peptide mapping and comigration have provided evidence that this is a neurofilament-like protein (Kelly and Cotman, 1977a; Yen, Kelly, Liem, Cotman and Shelanski, 1976; Blomberg *et al.*, 1977; Therien and Mushynski, 1976). Kelly and Cotman (1977b) have called this neurofilament-like protein 'synapsin'.

The PAGE results of Kelly and Cotman (1977a) suggest that there are possibly 9 polypeptide bands unique or enriched in PSD's as compared to SJC's. One component with a molecular weight of 97,000 daltons seems to be unique to PSD's (Banker *et al.*, 1974). Similarly Cohen *et al.* (1977) found 7 polypeptides to be unique for PSD's.

In a detailed study of the effects of salts, detergents and sulfhydryl-blocking agents on the structure of PSD's as monitored by PAGE and electron microscopy, Blomberg *et al.* (1977) concluded that the neurofilament-like protein forms the core of PSD's and that these filaments are cross-linked by smaller filaments (with subunits of 100,000 daltons) and by the actin-like protein. In addition, there are two other components which could be phosphorylated by ATP through a cyclic-AMP dependent protein kinase.

A postsynaptic membrane preparation, which preserves the outside or cleft regions, as opposed to PSD preparations which preserve the inside or cytoplasmic regions, has been found to be enriched in 3', 5' cyclic nucleotide phosphodiesterase, (Na^+-K^+)-ATPase, acetylcholinesterase, muscarinic and nicotinic receptors (Bartfai *et al.*, 1976). Immunohisto-

chemical studies have confirmed the localization of acetylcholinesterase (McBride and Cohen, 1972) and 3',5' cyclic nucleotide phophodiesterase (Florendo, Barrnett and Greengard, 1971) on the postsynaptic membrane.

The presence of a very large number of disulfide bonds in synaptic components as demonstrated by Kelly and Cotman (1976) suggests a critical role for these bonds in that " . . . tubulin, neurofilament protein and perhaps other fibrous proteins use intermolecular disulfide bonds to differentiate into the postsynaptic density matrix and tie into the membrane."

Thus two possible primary roles for the postsynaptic density emerge from the available data, forming a tentative picture of synaptic mechanisms. Firstly, the presence of tubulin, actin and ATPase suggests chemo-mechanical properties (Kelly and Cotman, 1977c). That a number of components can be phosphorylated through a cyclic AMP dependent kinase provides support for the hypothesis. "The PSD appears held together by disulfide bonds, hydrophobic interactions, and calcium coordination bonds, so that factors affecting these bonds would affect the static and dynamic properties of the PSD and its interaction with the neuron's cytoskeletal network." (Feit, Kelly and Cotman, 1977). Kelly and Cotman (1977c) suggest that presynaptic activity, may activate ATPase, possibly through changes in intracellular calcium and thus alter postsynaptic structure.

A second role for the PSD is that it may provide a cytoarchitectural base which restricts the distribution or lateral diffusion of components present on the postsynaptic membrane (Matus *et al.*, 1976; Cotman, 1976). This is supported by evidence indicating that Con-A receptors (Kelly, Cotman, Gentry and Nicolson, 1976; Matus, DePetris and Raff, 1973) and unidentified intramembraneous particles (Landis and Reese, 1974) present at the synapse are relatively immobile as compared to components present on adjoining plasma membrane.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

Triton X-100, a non-ionic detergent, was the product of Harmann-Ledden Co., Philadelphia, Pa. Agarose and Freund's adjuvant were obtained from Difco Co., Detroit, Mich. Ficoll 400, Sepharose 6B, and Sephadex were purchased from Pharmacia, Montreal, Canada. Bio-Gel P-150 was bought from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Canada. Acrylamide and N,N'-Methylene-bisacrylamide were purchased from Eastman Kodak Co., Rochester, N.Y.

Most of the proteins used as standards for molecular weight determinations were obtained from Sigma Chemical Co., St. Louis, Mo., as were most of the biochemicals used for enzyme assays and aprotinin. CDP-^{14}C-choline was bought from New England Nuclear (Canada, Ltd.), Toronto. The diacylglycerol was a generous gift from Dr. F. Possmayer.

All other chemicals were reagent grade.

Collodion membranes used for concentration by positive pressure dialysis were manufactured by Membranfiltergesellschaft, Göttingen, Germany.

Sheep red blood cells preserved in Alsever's solution were supplied by Sylab, Petrolia, Ont. Complement, lyophilized,

and kept at -20°C, was obtained from harvesting the serum from freshly bled Guinea pigs.

Wistar rats were supplied by Woodlyn Laboratories, Guelph, Ontario and female New Zealand White rabbits, 5-6 lbs were purchased from Riemens Fur Ranches, Ltd., St. Agatha, Ontario.

2.2 METHODS

2.2.1 Preparation of Subcellular Membranes and Organelles

Fasted adult Wistar rats (300g) of both sexes were sacrificed by decapitation, and the cerebral hemispheres were rapidly removed from the brains to prechilled containers, washed with 0.32M sucrose, weighed and placed in cold 0.32M sucrose.

2.2.1.1 Synaptosomes and RSM

Synaptosomes and RSM were prepared by the method of Cotman and Mathews (1971), as described in Figure 1, except that the P_2 pellet was "washed" according to Gurd *et al.*, (1974) by resuspending it in the original volume of 0.32M sucrose and recentrifuging at 15,600 rpm (17,000g$_{av}$) for 10 min. The purified RSM Fraction 2 (Figure 1) was used for the experiments described in this paper.

2.2.1.2 Brain microsomes (RBM)

The supernatant from the crude mitochondrial fraction (P_2) was centrifuged at 105,000g for one hour. The microsomal pellet was washed by resuspending it by hand homogenization in 20 times its volume of 0.32M sucrose and discarding the

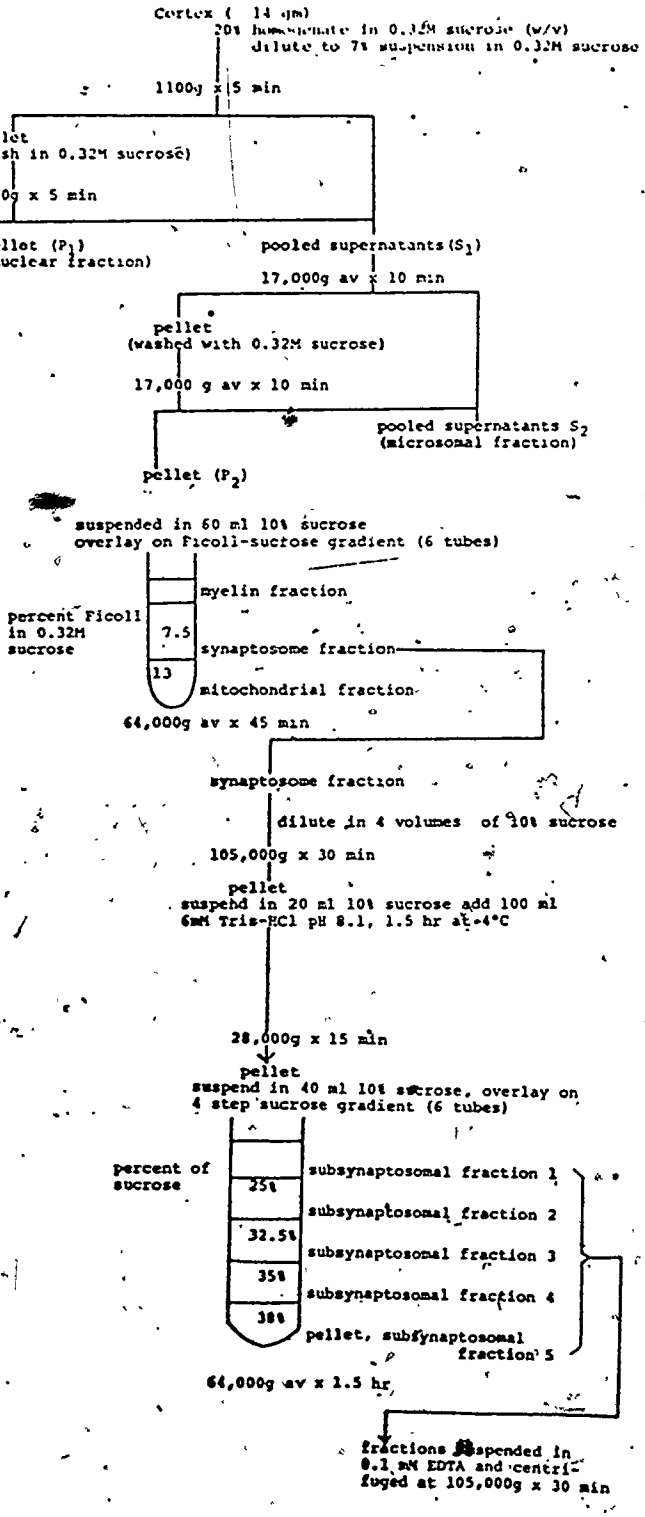


Figure 1. Flow sheet for the preparation of synaptic plasma membranes.

precipitate formed when the suspension was recentrifuged at 17,500g. RBM were then pelleted by centrifugation at 105,000g for one hour. The pellet was suspended in 0.32M sucrose at a concentration of 20 mg protein/ml and stored at -20°C.

2.2.1.3 Soluble fraction

The microsomal supernatant or 'soluble fraction' (RBE), MacPherson *et al.* (1973), was dialyzed against saline and concentrated by ultrafiltration under positive pressure to contain 30 mg of protein/ml and stored at -20°C.

2.2.2 Assays for Marker Enzymes

The mitochondrial marker, succinate dehydrogenase (EC 1.3.99.1) was determined according to the method of Laatsch, Kies, Gordon and Alvord (1962) and of Possmayer, Meiners and Mudd (1973), by measuring the succinate-dependent reduction of 2'-(p-iodophenyl)-3p-nitrophenyl-5-phenyl tetrazolium chloride. The reaction mixtures were incubated at 37°C for 10 min and the reaction stopped by the addition of 0.1 ml of 12N HCl. The formazan formed was extracted with 4 ml of ethyl acetate and the E_{492} of the ethyl acetate extracts were determined. A molar extinction coefficient of 20.1×10^3 litre $mol^{-1} \cdot cm^{-1}$ was used to calculate the amount of formazan formed. Results were expressed as n moles formazan formed per $min \cdot mg^{-1}$ protein.

Acetylcholinesterase (EC 3.1.1.7) activity was assayed according to the method of Ellman, Courtney, Andres and Featherstone (1960) by following the increase of yellow color (E_{412}) produced from thiocholine when it reacts with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB). The E_{412} increase

of blanks containing tris-HCl buffer, quinidine sulfate, DTNB, and subcellular fraction was followed for 10 min and subtracted from the E_{412} increase obtained after addition of acetyl thiocholine iodide. A molar extinction coefficient of 1.36×10^4 litre $mol^{-1} \cdot cm^{-1}$ was used to calculate the specific activity in n moles substrate hydrolyzed per $min \cdot mg^{-1}$ protein.

NADPH: cytochrome c reductase (EC 1.6.2.3) was measured by the procedure of Sottocasa, Kuylenstierna, Ernster, and Bergstrand (1967). Blanks were composed of 60 mM K_2HPO_4-KH_2PO_4 buffer, pH 7.5, 0.3 mM KCN, 0.075 mM cytochrome c, 0.0025% Nonidet P40 and from 0.04 to 0.16 mg protein (subcellular fraction). The E_{550} was recorded for 10 min. The reaction was initiated by adding enzymatically reduced NADPH and the E_{550} was recorded for 10 min. A molar extinction coefficient of 19.1×10^3 litre $mol^{-1} \cdot cm^{-1}$ was used to calculate the reduction of cytochrome c. Results were expressed as n moles cytochrome c reduced per $min \cdot mg^{-1}$ protein.

CDP-choline diacylglycerol cholinephosphotransferase (EC 2.7.8.2) was determined as described by Possmayer *et al.* (1977). The reaction mixture contained 100 mM Tris-HCl, pH 7.4, 1.0 mM EDTA, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 8-12 mM diacylglycerol suspended in Tween 20, 0.5 mM CDP-(Me-^{14}C)-choline (0.6 µCi/µ mol), and 0.05-0.1 mg enzyme preparation. After incubation at 37°C for 30 min, the reaction was halted by the addition of 10 ml chloroform-methanol (1:1) and the lipid extracts purified as described by Possmayer and Mudd

(1971). Radioactivity was determined after suspending the lipids in 5 ml of toluene containing 0.5%, 2,5-diphenyloxazole. The enzyme assays were carried out under conditions for which the rates were linear with enzyme concentration.

2.2.3 Protein determination

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard. Protein of membrane fractions was determined by incubating the fraction (suspended in 1N NaOH) in a boiling water bath for 10 min. Similarly treated bovine serum albumin was used as standard.

Protein of Triton X-100 extracts was determined by a modification of the procedure of Sugawara (1975). Triton extracts (0.1 ml), diluted so as to contain 10% or less of Triton X-100, were added to 0.4 ml 20% SDS and 0.5 ml water and then protein determined according to the method of Lowry *et al.* (1951). BSA suspended in 0.1 ml 10% Triton X-100, 0.4 ml 20% SDS and 0.5 ml water was used as a standard.

2.2.4 Removal of extrinsic membrane proteins and ribosomes by "washing"

One milliliter aliquots of freshly prepared RBM or RSM containing 30 mg of protein were homogenized in succession in 30 vol of (1) 0.14M NaCl, (2) 1.0M NaCl, (3) 0.1M $NaHCO_3$-Na_2CO_3 in a modification of the washing procedure used for microsomes by Hinman and Philips (1970). After each homogenization, the suspension was allowed to stand for 30 min and then centrifuged at 105,000 g for one hour. The

supernatant "salt washings" were concentrated by ultrafiltration.

The "washed" RBM will hereafter be referred to as endoplasmic reticulum (RBER) to distinguish them from "unwashed" RBM.

The pellets of RBER and "washed" RSM were washed finally with distilled water, pelleted as above and stored at -20°C.

2.2.5 Solubilization of Membranes

2.2.5.1 Differential extraction with non-ionic detergent, Triton X-100

Three different procedures for Triton X-100 solubilization of membranes as outlined below, were used for this thesis.

Method 1. Originally, aliquots of "washed RSM" or RBER containing 30 mg of protein were suspended in 100 ml of 1% Triton X-100 in 0.01M Tris-HCl, pH 7.4, homogenized in a Teflon-glass homogenizer (clearance 0.25 mm) with three up-and-down passes at a speed of 2,000 rpm. After incubation at 37°C for one hour, the suspensions were centrifuged at 105,000g for one hour. The supernatants were concentrated by positive pressure dialysis in collodion membranes to about 5 ml and labelled TxO.

Method 2. Two ml of "washed RSM" or RBER containing 20 mg of protein were added to 2 ml of 4% Triton X-100 in 0.1M Tris-HCl, pH 7.4, to give a final concentration of 2% Triton X-100, and homogenized as above, the supernatants were

concentrated to 1 ml on a rotary evaporator at 37° and labelled TxA. The pellet was then homogenized with 100 ml of 1% Triton X-100. After incuabtion and centrifugation as above, the supernatants were concentrated to about 4 ml and labelled TxB.

Method 3. Aliquots of "washed RSM" or RBER containing 20 mg of protein were suspended in 16 ml of 2% Triton X-100 in 0.01M Tris-HCl, pH 7.4 and homogenized as above. After incubation and centrifugation as above, the supernatants, labelled Tx1a were concentrated to 1 ml on a rotary evaporator. The pellet was reextracted with 16 ml of 2% Triton X-100 as above, and the concentrated extracts were labelled Tx1b. The remaining pellet was finally homogenized with 100 ml of 1% Triton X-100. After incubation and centrifugation, the supernatants were concentrated to about 4 ml and labelled Tx2.

The Triton X-100 extracts were used as such or Triton X-100 was removed by one of the following procedures.

Procedure 1. Triton X-100 extracts were lyophilized and washed 4 times with 30 ml of n-butanol and 2 times with 30 ml of acetone. The remaining precipitate was solubilized in water.

Procedure 2. Triton X-100 extracts were added to 9 volumes of acetone and the precipitate formed was solubilized in water.

2.2.5.2 Extraction with butanol

Aliquots of "washed RSM" or RBER containing 10 mg of protein were suspended in 4 ml of cold water and 10 ml of cold butanol and homogenized with 3 up-and-down passes in a Teflon-glass homogenizer. The suspension was vigorously mixed

for 20 seconds and allowed to stand at 4°C for 15 min. The emulsion was then centrifuged at 23,000g for 15 min. The water phase was removed with a syringe and the butanol layer and insoluble fluff were homogenized and shaken as above with 4 ml of distilled water. After standing for 15 min the emulsion was centrifuged at 37,000g for 15 min. The pooled water phases were either concentrated by positive pressure dialysis in collodion membranes or after addition of 0.2 ml of 1% Triton X-100, concentrated by flash evaporation to about 0.5 ml.

2.2.6 Purification and Molecular Weight Determination of Antigens X1 and X2 by Gel Filtration

Calibrated columns of Sephadex, Sepharose 6B or Biogel P-150 were used for the partial purification and molecular size estimations of antigens X1 and X2. The columns were packed and calibrated in the manner described by Andrews (1964) using proteins of known molecular weight as standards. The void volume was determined using Blue Dextran 2000. The eluting buffers, elution rates, and preparation of samples will be presented later (see Results).

2.2.7 Polyacrylamide Gel Electrophoresis

Polyacrylamide disc gel electrophoresis was performed according to the method of Davis (1964) except that the sample and spacer gels were omitted. The sample, containing 50 to 200 µg of protein in approximately 0.05-0.1 ml was applied directly on top of the running gel (pH 8.9) and the electrode buffer was carefully layered over the sample.

A constant current of 2 ma per tube was applied until the bromophenol blue dye had entered the gel; then the current was increased to 8 ma per tube until the dye had travelled 6 cm in the gel. Gels were removed from their tubes, stained with 0.25% Coomassie blue in 7% acetic acid for two hours. The gels were destained electrophoretically in 7% acetic acid.

Separation of proteins according to molecular size was performed according to the method of Weber and Osborne (1969), by polyacrylamide gel electrophoresis in the presence of SDS. Either 10 or 12% polyacrylamide containing 0.1M phosphate buffer, 0.1% SDS, 0.045 ml TEMED, and 0.075% ammonium persulfate were used. Samples were either incubated directly in dialysis buffer for 30 min or were mixed with 2-mercaptoethanol and/or SDS (to final concentrations of 5% and 1% respectively), incubated in a boiling water bath for 3 min and then incubated with dialysis buffer for 30 min. Three µl of 0.05% bromophenol blue and one drop of glycerol were added to the sample and the mixture applied directly to the top of the gels and the electrode buffer was carefully layered on top of each sample to fill the tubes. A constant current of 8ma per gel was applied until the marker dye had moved 6 cm through the gel (4-5 hrs). The gels were fixed (5% methanol and 9% acetic acid) overnight, stained for 3 hours with 0.25% Coomassie blue (in 5% methanol and 9% acetic acid), and then destained in several changes of 7.5% acetic acid-5% methanol. For

molecular weight determinations, the mobility of proteins was calculated using the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against known molecular weights (standards) expressed on a semilogarithmic scale.

Glycoproteins were stained by the periodic acid-schiff method of Zacharius *et al.* (1969).

2.2.8 Preparation and Absorption of Antisera

Anti-RSM sera were raised in rabbits by injecting them initially in the hind footpads with 3.0 mg of RSM protein (subsynaptosomal fraction F_2) emulsified in complete Freund's adjuvant. Injections were repeated intramuscularly every 3 to 4 weeks for several months. Animals were bled regularly from the ear vein when the level of antibodies directed against immunogenic intrinsic membrane components was satisfactory to carry out immunodiffusion studies. All of the satisfactory bleedings from one rabbit that had made a typical response to immunization with RSM were pooled to form the anti-RSM serum A used for this study.

Anti-"washed" RSM was raised by rabbits in similar fashion and all satisfactory bleedings from one rabbit were pooled to form the anti-RSM serum B used for this study.

Anti-RBM serum A was raised by rabbits in similar fashion and consisted of the pooled sera from six rabbits. Anti-RBER serum B consisted of the pooled bleedings of a rabbit immunized for several months with "washed" RBM.

Antibodies to rat serum proteins, brain soluble fraction proteins and extrinsic membrane proteins were absorbed from the anti-RSM and anti-RBER sera by mixing 1.0 ml of RBE containing 30 mg of protein/ml with 5.0 ml of antiserum at 37°C for 1 hour. The specific precipitates were removed by centrifugation and aliquots of the absorbed sera were concentrated to one-half the original volume by ultrafiltration and tested by immunodiffusion analyses for the presence of antibodies of RBE. The absorption process was repeated until all the antibodies that reacted with RBE components had been removed. Crude globulin fractions (hereafter referred to as 'IgG' fractions) of the sera absorbed with RBE were prepared by precipitation of the globulins by 40% saturation with ammonium sulfate. The precipitates were dissolved in 0.9% saline at one third the original volume and dialyzed against 0.9% saline under positive pressure until free from ammonium ions.

2.2.9 Immunochemical Analyses

2.2.9.1 Double diffusion analyses

Ouchterlony (1953) type analyses were carried out on glass microscope slides. The slides were covered with agarose (1.2%)dissolved in 0.05M barbiturate buffer pH 8.6 and 1% Triton X-100. The wells were cut in the solidified gel in a suitable pattern and, antigen solution and antiserum were added to the appropriate wells. The plates were left at room temperature for 24 hours, and the results were recorded and photographed. After washing for 3 days in frequent changes of saline, the slides were dried and stained with acid fuchsin.

Aprotinin (5%) was added to the agarose for some analyses in order to facilitate the diffusion of antigen X1 from the antigen wells.

2.2.9.2 Immunoelectrophoresis

Immunoelectrophoresis was carried out according to the method of Wieme and Rabaeye (1957), on glass microscope slides coated with a 1.2% solution of agarose containing 0.05M barbiturate buffer pH 8.6 and 1% Triton X-100, using a constant current of 15 mA per slide for 45 min.

2.2.9.3 Localization of antigens after electrophoresis in polyacrylamide gels

After completion of the electrophoresis in SDS polyacrylamide gels, the position of antigens was located by placing the gel in a slit cut out from a slab of buffered agarose containing 1% Triton X-100. Melted agarose was then carefully poured around the gel so as to remove all air bubbles. A second trough was cut alongside the polyacrylamide gel and filled with antiserum. After 48 hr at room temperature, the mid-point of precipitin arcs was taken to indicate the position of the antigen in the gel.

2.2.10 Quantitation of X2 by Single Radial Immunodiffusion

The quantitation of X2 by single radial immunodiffusion was carried out according to the method of Mancici, Carbonara and Heremans (1965). Anti-RBER serum B (0.8 ml), heated in a water bath at 55°C, was added to 9.2 ml of 1.2% agarose in barbiturate buffer (containing 1% Triton X-100), held at the same temperature. The mixture was pipetted onto a warm glass

plate (7.5 x 5 cm). After the gel had solidified, rows of wells spaced 1.5 cm apart were punched out, using a No. 12 hypodermic needle from which the bevel had been cut off. Measured volumes of Triton X-100 extracts, containing known amounts of protein, were added into the wells. The plates were stored in a moist chamber at room temperature for 24-48 hours. To obtain the standard calibration line, the diameters of the circles formed around the wells containing an RBM Txla extract (five different volumes), were measured and plotted on the abscissa against the concentrations (arbitrary units) of the RBM Txla extracts on the ordinate. Concentrations of X2 in the other Triton X-100 extracts were calculated from the standard calibration graph using ring diameters formed by the respective solutions.

2.2.11 Complement Fixation Technique

The microcomplement fixation test was performed using the microtiter system supplied by Cooke Engineering Co., Alexandria, Virginia. The technique of Sever (1962) was used. Modified barbiturate buffer was prepared according to the method of Mayer *et al.* (1946).

Sheep erythrocytes were washed by suspending 10 ml of sheep's blood (in Alsever's solution) in 30 ml of isotonic saline and centrifuging at 1,500 rpm for 10 min. This washing process was repeated twice. The erythrocytes were diluted in buffer and the suspension adjusted to contain 5% erythrocytes by colorimetric standardization (E_{550} of a 5% erythrocyte suspension is 0.56).

Hemolysin was titrated by setting up 0.1 ml of two-fold serial dilutions of stock of anti-sheep red blood cell hemolysin in a series of test tubes. To each tube was then added 0.1 ml of 5% sheep erythrocytes, 0.4 ml of buffer, and 0.2 ml of guinea-pig complement diluted 1:20. After mixing, the tubes were incubated at 37°C for 30 min, centrifuged, and the E_{550} of the supernatants were measured. The highest dilution of hemolysin giving complete hemolysis was taken as one unit. For sensitization of sheep erythrocytes in subsequent experiments, four units of hemolysin were used.

Lyophilized guinea-pig complement (stored at -20°C in 1 ml samples) was titrated by setting up a series of test tubes each containing 1 ml of a complement dilution ranging from 1:10 to 1:500. To each tube was added 1 ml of sensitized sheep erythrocytes and 5.5 ml of buffer. After mixing, the tubes were incubated at 37°C for 30 min, centrifuged and the E_{550} of the supernatants measured. The amount of complement required to produce 50% hemolysis was determined and taken as one 50% hemolytic unit.

The optimal concentration of antigen was established by performing the fixation test with a wide range of dilutions (1:100 to 1:1,600) of RBM or RSM. The antigen concentration which gave the highest complement fixing titre when added to serial, two-fold dilutions of the antiserum (anti-RSM serum A), was considered optimal and used in subsequent experiments. In this study, the optimal antigen dilution was 1:100 of a membrane preparation containing 5 mg protein per ml.

The measurement of the complement fixing titre of the antiserum was performed in disposable plastic plates containing U-shaped wells. All antisera were absorbed with an equal volume of washed, packed sheep erythrocytes and inactivated at 56°C for 20 min. Two-fold serial dilutions of antisera (25 µl) were set up in the wells. To each well was added 25 µl of diluted antigen and 50 µl of complement containing three 50% hemolytic units. The plates were gently shaken and incubated at 37°C for 30 min. Sensitized sheep erythrocytes (25 µl) were then added to every well and after gentle shaking the plates were incubated at 37°C for 30 min. The following controls were used: (1) antiserum + complement + sensitized cells; (2) antigen + complement + sensitized cells; (3) complement + sensitized cells, and (4) antiserum + antigen + sensitized cells. The titres of the antisera were expressed as the reciprocal of the highest dilution of antisera which did not produce complete hemolysis.

2.2.12 Synaptosome Swelling Assay

The synaptosome swelling assay as described by Kaiteri *et al.* (1972, 1974) was used to measure ISS antibody in the anti-RSM serum A and anti-RBM serum A. Synaptosome suspensions (40 µl) were mixed with 20 µl of normal rabbit serum (NRS) and 30 µl of 0.32M sucrose and incubated for 10 min at 25°C. Guinea pig complement (50 µl) was added and the mixture incubated for 50 min at 25°C. Fifty µl of the reaction mixture was then added to 400 µl of 0.4M glycerol in a

microcuvette, mixed rapidly and the decrease in E_{520} was followed for 4 min. The synaptosome suspension was diluted with 0.32M sucrose so as to give a decrease of 0.3 optical density units and this E_{520} decrease is taken as 100% swelling.

When testing antibody activity, the swelling test was repeated as above, by using various volumes of antiserum in place of NRS. The volume of 0.32M sucrose was adjusted so that each tube contained 90 µl of reaction mixture. Antibody activity was detected as a smaller E_{520} decrease than was obtained with NRS. The results were expressed as percentage inhibition of synaptosome swelling (ISS).

The antisera were absorbed by the addition of membrane fraction, incubation at 37°C for 30 min, followed by overnight incubation at 4°C. The suspensions were then centrifuged at 105,000g for one hour.

2.2.13 Electron Microscopy

RSM fraction F_2 and synaptosomes were fixed with 5% glutaraldehyde (in 0.05M Na-cacodylate-HCl, pH 7.4), post-fixed with 1% Osmium tetroxide, stained with 1% aqueous uranyl acetate, dehydrated, and embedded in propylene oxide: araldite (1:1).

CHAPTER 3. RESULTS

3.1 ENZYME ASSAYS AND ELECTRON MICROSCOPY

The method of Cotman and Mathews (1971) was used to prepare synaptic membranes because this method yields working quantities of purified synaptic membrane protein with minimal contamination by myelin, glial membranes and mitochondria. The procedure as described by Cotman and Mathews was modified slightly : (1) the nuclear pellet was washed once with 0.3M sucrose and the supernatant was pooled with S_1, and (2) the crude mitochondrial pellet was washed once with 0.32M sucrose and the supernatant was pooled with S_2.

A number of reports had demonstrated the importance of washing the P_2 fraction in order to reduce microsomal contamination (Morgan *et al.*, 1971; Gurd *et al.*, 1974; Levitan *et al.*, 1972). Gurd *et al.* (1974) had indicated that three washes with 0.32M sucrose decreased the microsomal contamination (as measured by RNA and NADPH : cytochrome c reductase) of the P_2 fraction from 50% (unwashed P_2) to less than 15%. Examination of the results reported by Gurd *et al.* (1974) indicated that most of the microsomal contamination is removed by the first wash of the P_2. Therefore, to maximize the yield of SM, it was decided to wash the crude mitochondrial fraction only once.

The microsomal fraction is defined in operational terms as the membrane material that sediments between 17,000 x g and 105,000 x g from cell homogenates in 0.32M sucrose

(De Robertis *et al.*, 1962). Since this fraction contains substantial amounts of small synaptosomes (Kataoka and De Robertis, 1967), it was decided to further purify the microsomal fraction (RBM) by resuspending the RBM in its original volume of 0.32M sucrose and recentrifuging at 17,000xg for 10 min. The pellet was discarded and the supernatant was pelleted at 105,000xg for one hour to obtain the RBM used for the present study.

Cotman and Mathews (1971) had demonstrated by electron microscopy and by enzyme markers, that the subsynaptosomal fraction F_2 contained the purest fraction of SM. Therefore, this fraction was used as the purified synaptic membrane for the immunological studies to be reported.

To ascertain whether the preparations of synaptic membranes were comparable to those reported in the literature, the yields of the major subcellular fractions as well as the relative specific activities of selected marker enzymes, in the fractions, were determined (Table 1).

The yield of 1.2 mg of SM protein (F_2) per gram of fresh brain cortex (wet weight) is lower than that of Cotman and Mathews (1971) who reported a yield of approximately 2 mg per gram cortex. This difference may be the result of the washing of the P_2 fraction prior to the separation of synaptosomes. In this regard, Jones and Matus (1974) reported that the yield reported by Cotman and Mathews is seldom reached. Gurd *et al.* (1974) obtained a yield of 200-250 µg

TABLE 1. Distribution of protein and enzyme activities in subcellular and subsynaptosomal fractions prepared from rat brain cortex.

FRACTION	PROTEIN[a]	MARKER ENZYMES[b]			
		NADPH: cytochrome c reductase	Choline phospho-transferase	Acetyl cholin-esterase	Succinic dehydrogenase
Homogenate	99.2	3.74 (4)[c]	0.77 (6)	79.0 (4)	28.7 (4)
Nuclear	14.1	0.75 (2)	1.04 (6)	0.35 (4)	0.4 (3)
Microsomes	10.4	2.03 (4)	4.20 (6)	2.13 (4)	0.1 (4)
Soluble	23.8	0.80 (2)	0.13 (6)	1.41 (4)	0.2 (4)
P_2	40.4	0.81 (3)	0.36 (6)	0.59 (4)	2.3 (3)
Crude Myelin	17.2	0.17 (2)	0.39 (3)	0.47 (4)	0.5 (3)
Mitochondria	12.2	0.89 (2)	0.17 (3)	0.64 (4)	3.0 (3)
Synaptosomes	7	0.89 (4)	0.22 (3)	0.70 (4)	2.1 (3)
Subsynaptosomal F_1	0.9	0.26 (4)	0.34 (3)	0.77 (4)	0.1 (4)
Subsynaptosomal F_2	1.2	0.77 (4)	0.40 (3)	1.61 (4)	0.1 (3)
Subsynaptosomal F_3	0.5	0.67 (2)	0.33 (3)[d]	0.92 (4)	0.9 (3)
Subsynaptosomal F_4	0.4	1.17 (2)		0.54 (4)	0.9 (3)
Subsynaptosomal F_5	1.4	0.42 (2)	0.23 (3)	0.33 (4)	1.8 (3)

The results represent the average of two to six experiments.

(a) Protein is listed as mg/g wet weight of cortex.

(b) Enzyme marker activities are expressed as relative specific activities by assigning a value of 1 to the specific activity of the homogenate. The results for the homogenate are expressed as specific activity. Specific activity = µmol substrate produced or utilized per min/mg protein.

(c) The number of assays is given in brackets.

(d) Average of subsynaptosomal fractions F_3 and F_4.

of SM proteins per gram cortex.

The relative specific activity (RSA) of the inner mitochondrial membrane marker, succinate dehydrogenase (SDH), in the subsynaptosomal fraction F_2 was 0.1 (Table 1). This is lower than the value for cytochrome oxidase as reported by Cotman and Mathews (1971).

The RSA of acetylcholinesterase in the subsynaptosomal fraction F_2 was almost twice that of the F_1 and F_3 indicating that this enzyme had been concentrated in the SM fraction. It can be seen that the microsomal fraction and the soluble fraction had RSA's of 2.1 and 1.4 respectively. Kokko (1969) and Novikoff (1967) demonstrated by histochemical methods that AChE is present in the endoplasmic reticulum, plasma membrane, and axoplasm. Aldridge and Johnson (1959) had reported that the microsomal fraction has the highest specific activity for AChE. This enzyme is therefore not a specific plasma membrane marker but it is useful to monitor the distribution of plasma membrane in the subsynaptosomal fractions. In the present study, approximately 39% of the recoverable synaptosomal AChE was localized in F_2. This is comparable to the results of Cotman and Mathews (1971).

Table 1 lists the RSA's of NADPH: cytochrome c reductase and CDP-choline diacylglycerol cholinephosphotransferase for the various fractions. The RSA of F_2 for NADPH: cytochrome c reductase was about 38% of the value for the microsomal fraction. This is comparable to the microsomal

contamination of the A-1 fraction reported by Gurd *et al.* (1974) and by Van Leeuwen *et al.* (1976). A large proportion of the microsomal membranes present in the synaptosome fraction should localize in the subsynaptosomal fraction F_1. It will be seen from Table 1 that F_1 had an RSA of only 0.26. For this fraction, there is a significant non-linear increase in extinction in the absence of NADPH. Presumably, some substance present in F_1 directly reduces cytochrome c. Therefore, the extinction observed in the presence of NADPH is subject to a large error. We did not investigate the possibility that cytochrome c may have become a limiting substrate after addition of NADPH.

In contrast to the NADPH: cytochrome c reductase results, it will be seen from Table 1 that the RSA for cholinephosphotransferase in F_2 was only about 10% of the value obtained for the microsomal fraction. The results also indicate that there has been a 4 fold increase in cholinephosphotransferase specific activity in the microsomal fraction as compared to the homogenate. For NADPH: cytochrome c reductase the increase in activity was only 2 fold in the microsomal fraction. Cholinephosphotransferase would thus seem to be a more appropriate indicator of microsomal contamination in the subcellular fractions.

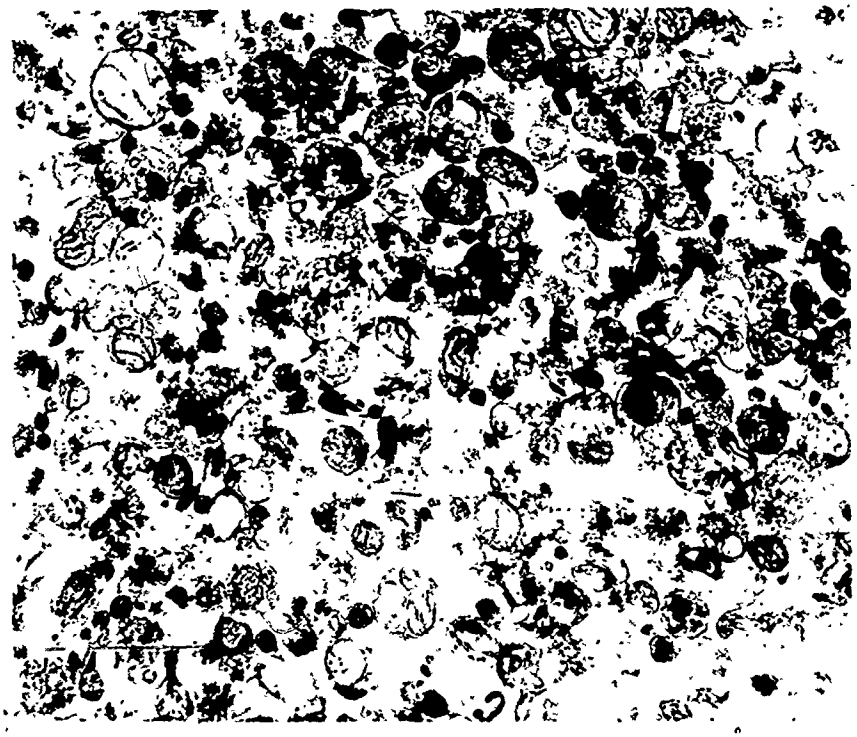
The synaptosomal fraction and the subsynaptosomal fraction F_2 were examined by electron microscopy (Figure 2). Examination of the synaptosomal fraction revealed the presence

Figure 2. Electron micrographs of

(A) the synaptosome fraction, and

(B) the subsynaptosomal fraction F_2.

A



B

of a large number of synaptosomes, some of which contain intraterminal mitochondria. A few free mitochondria, and a number of unidentifiable membranous elements can also be seen. The F_2 fraction contains sheets of membrane and vesicles. The general appearance of the synaptosomal fraction and the F_2 fraction was comparable to the electron micrographs presented by Cotman and Mathews (1971), Morgan *et al.* (1971), and Gurd *et al.* (1974).

3.2 SYNAPTOSOME SWELLING

We investigated the reliability of the method devised by Raiteri *et al.* (1972), which was reported to yield quantitative measurements of the concentration of antibody directed against synaptosomal surface antigens.

When synaptosomes are added to 0.4M glycerol there is a decrease in the optical density (O.D.) of the suspension when the O.D. is measured at 520 nm. This is assumed to be due to the swelling of synaptosomes in the hypotonic glycerol solution. To standardize the synaptosome preparation, the protein concentration was adjusted in order to obtain a decrease of 0.3 O.D. units with synaptosomes previously incubated with 20 µl of normal rabbit serum in the presence of guinea pig complement. This concentration was found to be 6.03 mg per ml of synaptosomal protein.

When synaptosomes were incubated with volumes of normal rabbit serum ranging from 2.5 to 40 µl, and then

placed in 0.4M glycerol, the extent of swelling decreased in linear fashion. This pattern was observed with three different pools of normal rabbit serum. The decrease in OD_{520}, obtained with synaptosomes incubated in normal rabbit serum, is taken as 100% swelling. Therefore, after incubation of synaptosomes with antisera, the OD_{520} decrease measured is adjusted to correct for decreased swelling that occurs after exposure to increasing volumes of normal serum.

In the initial studies, an IgG preparation of anti-RSM serum A was tested to see if the antibodies it contained would induce inhibition of synaptosomal swelling (ISS). It will be seen from Figure 3 that a maximum ISS of about 67% was obtained when 10 µl of anti-RMS IgG was used. Less ISS antibody activity was obtained with smaller volumes of IgG.

It will be seen from Figure 3 that the pattern obtained with anti-RSM serum A is similar to the curve obtained with anti-RSM IgG, except that a larger volume of serum was required to produce maximum ISS. Twenty µl of antiserum induced a maximum ISS of about 70%. When the swelling of synaptosomes, incubated with anti-RSM serum in the absence of complement, was measured, no ISS antibody activity was detected. Since the ISS activity was similar for anti-RSM serum and for anti-RSM IgG, it was decided to use anti-RSM serum for synaptosome swelling experiments.

Figure 4 demonstrates the ISS patterns obtained with an anti-RSM serum after it had been absorbed with various

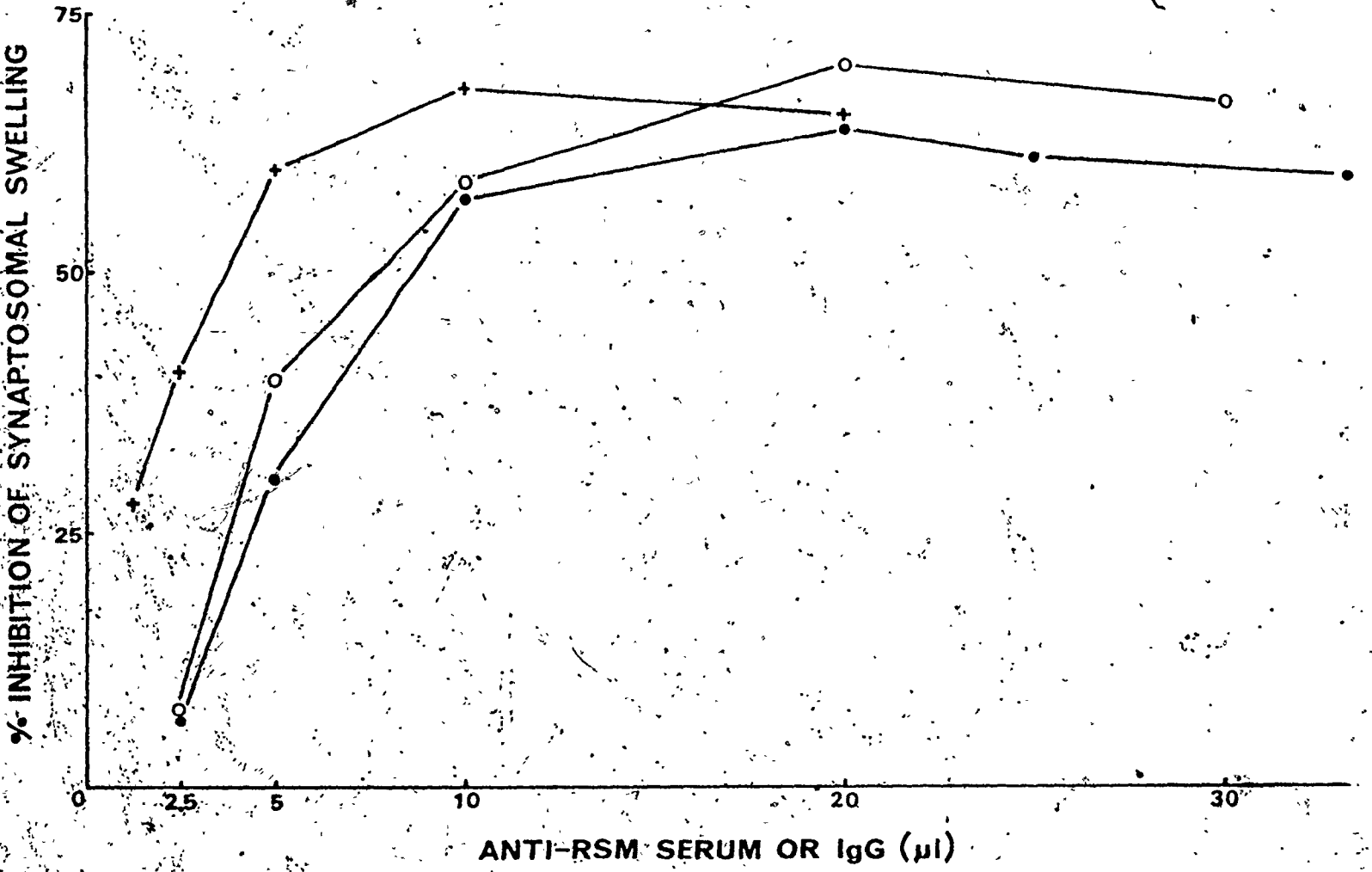
Figure 3. Inhibition of synaptosome swelling by anti-RSM serum and anti-RSM IgG, and effect of absorption of the serum with RBE.

The serum and IgG were prepared from anti-RSM serum A.

+———+indicates anti-RSM IgG.

o———oindicates anti-RSM serum.

•———•indicates anti-RSM serum absorbed with 15 mg RBE/ml serum.


% INHIBITION OF SYNAPTOSOMAL SWELLING
75
50
25
0
2.5
5
10
20
30
ANTI-RSM SERUM OR IgG (µl)

quantities of RSM. It will be seen that the antibody which causes inhibition of synaptosome swelling may be removed effectively from the serum. A gradual decrease of ISS antibody occurred up to the point where almost no antibody activity could be detected with serum that had been absorbed with 6 mg of RSM protein.

Absorption of anti-RSM serum with synaptosomes decreased the ISS antibody activity (Figure 5), but per mg of protein synaptosomes are not as effective as RSM in removing the ISS antibody. For example, absorption with 8 mg of synaptosomal protein removed only about half as much antibody as did absorption with 8 mg of RSM protein.

In Figure 6 are shown the curves obtained when anti-RSM serum was absorbed with increasing amounts of RBM. It will be seen that there was a fairly regular decrease of ISS activity with increasing amounts of RBM until 6 mg of RBM protein had been used; thereafter larger amounts of RBM protein produced only slight decreases in ISS activity. It will be noted that not all of the ISS antibody could be removed by RBM absorption. Thus, there seems to be approximately 5% of the ISS remaining after absorption with 15 or 20 mg of RBM.

Since a maximum of approximately 70% of the total synaptosomal swelling can be inhibited by unabsorbed anti-RSM serum, expression of the results in terms of actual antibody activity would give a clear picture of the comparative effects of various absorbents. The swelling obtained with 0.2 ml of unabsorbed serum was therefore expressed as 100% antibody

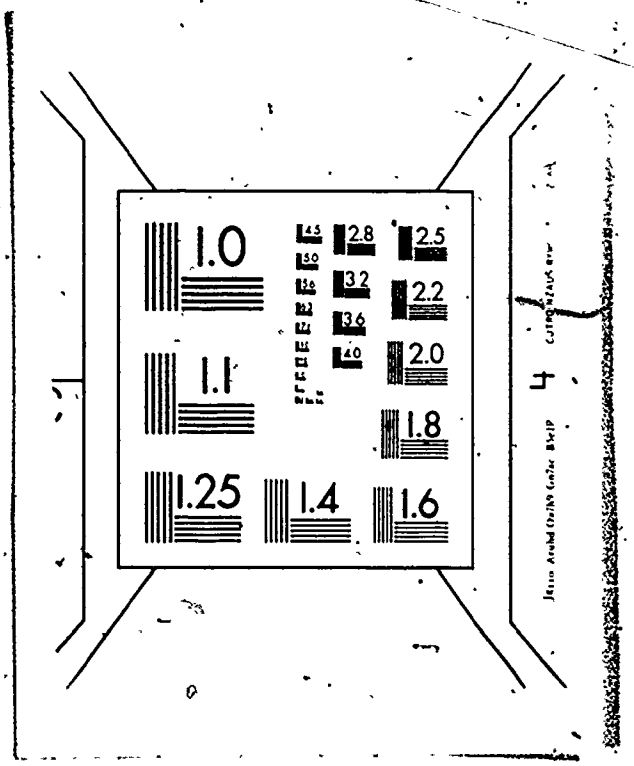

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6

Figure 4. Decrease in capacity of anti-RSM serum to inhibit synaptosomal swelling after absorption with different amounts of RSM protein.

The different amounts of RSM protein used to absorb the serum are indicated alongside each curve (in mg RSM protein/ml serum).

o———o indicates unabsorbed serum,

•———• indicates serum absorbed with RSM.

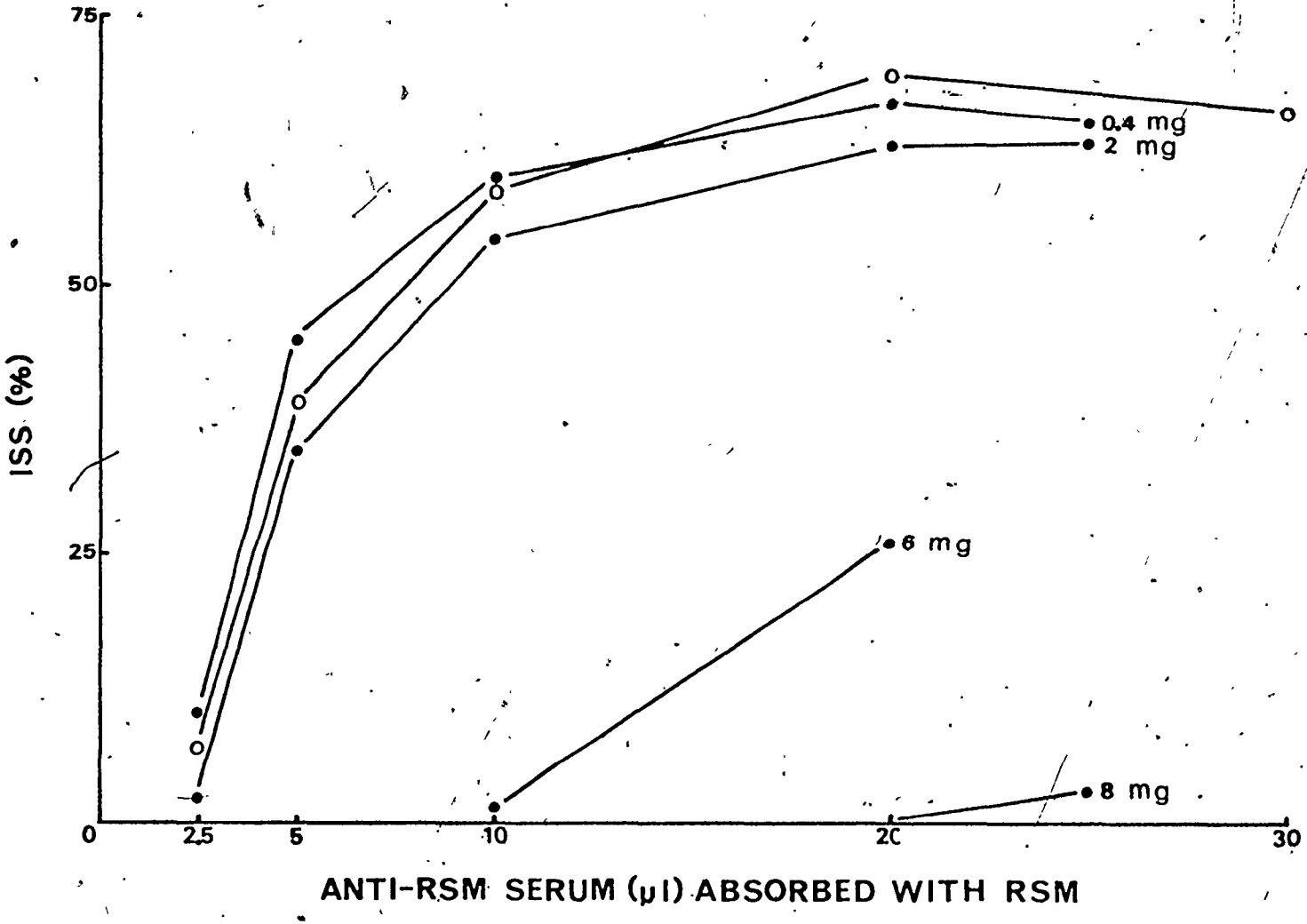

75
50
25
0
ISS (%)
0.4 mg
2 mg
6 mg
8 mg
2.5
5
10
20
30
ANTI-RSM SERUM (µl) ABSORBED WITH RSM

Figure 5. Decrease in capacity of anti-RSM serum to inhibit synaptosomal swelling after absorption with different amounts of synaptosomal protein.

The different amounts of synaptosomal protein used to absorb the serum are indicated alongside each curve (in mg synaptosomal protein/ml serum).

o——o indicates unabsorbed serum.

•——• indicates serum absorbed with synaptosomes.

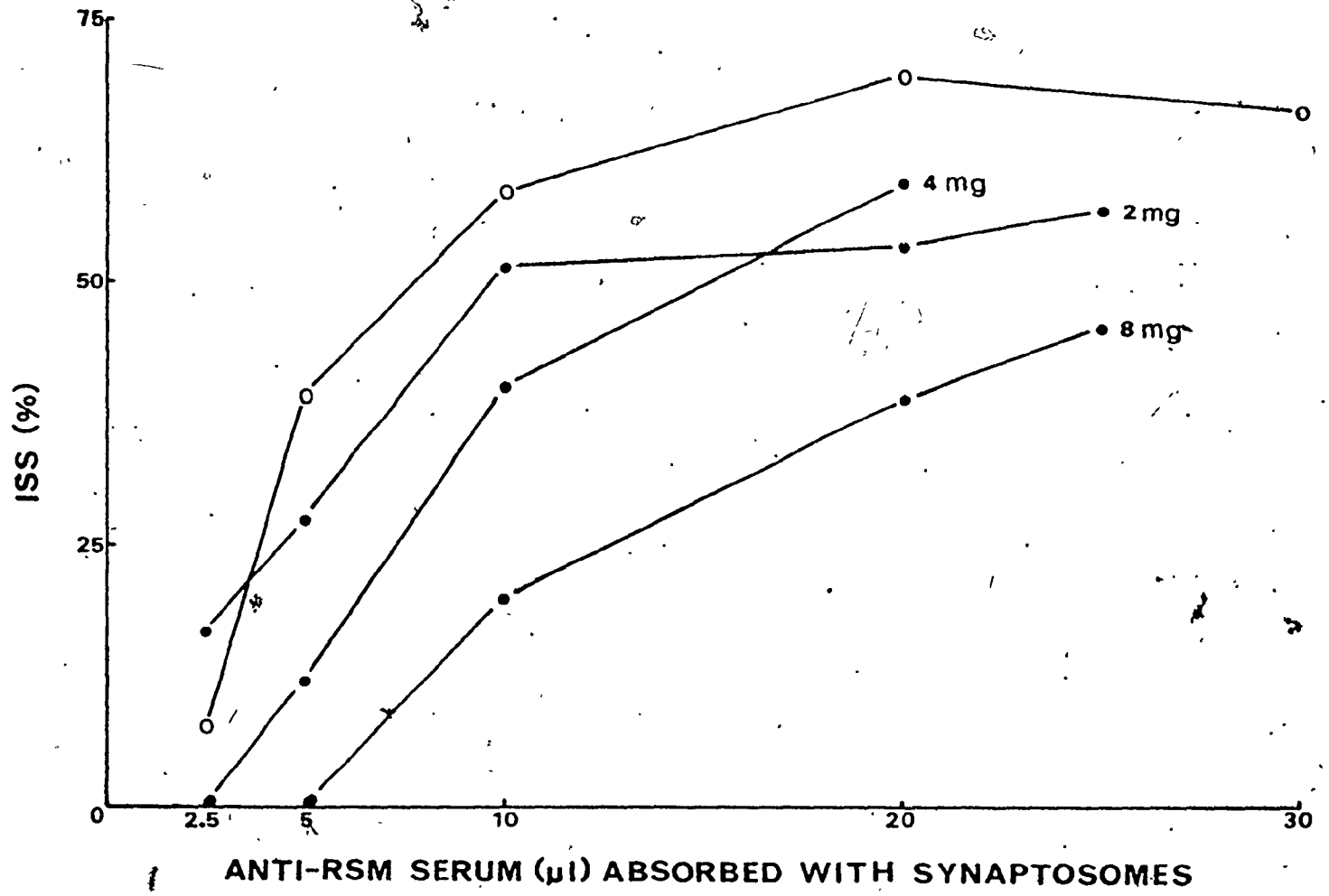
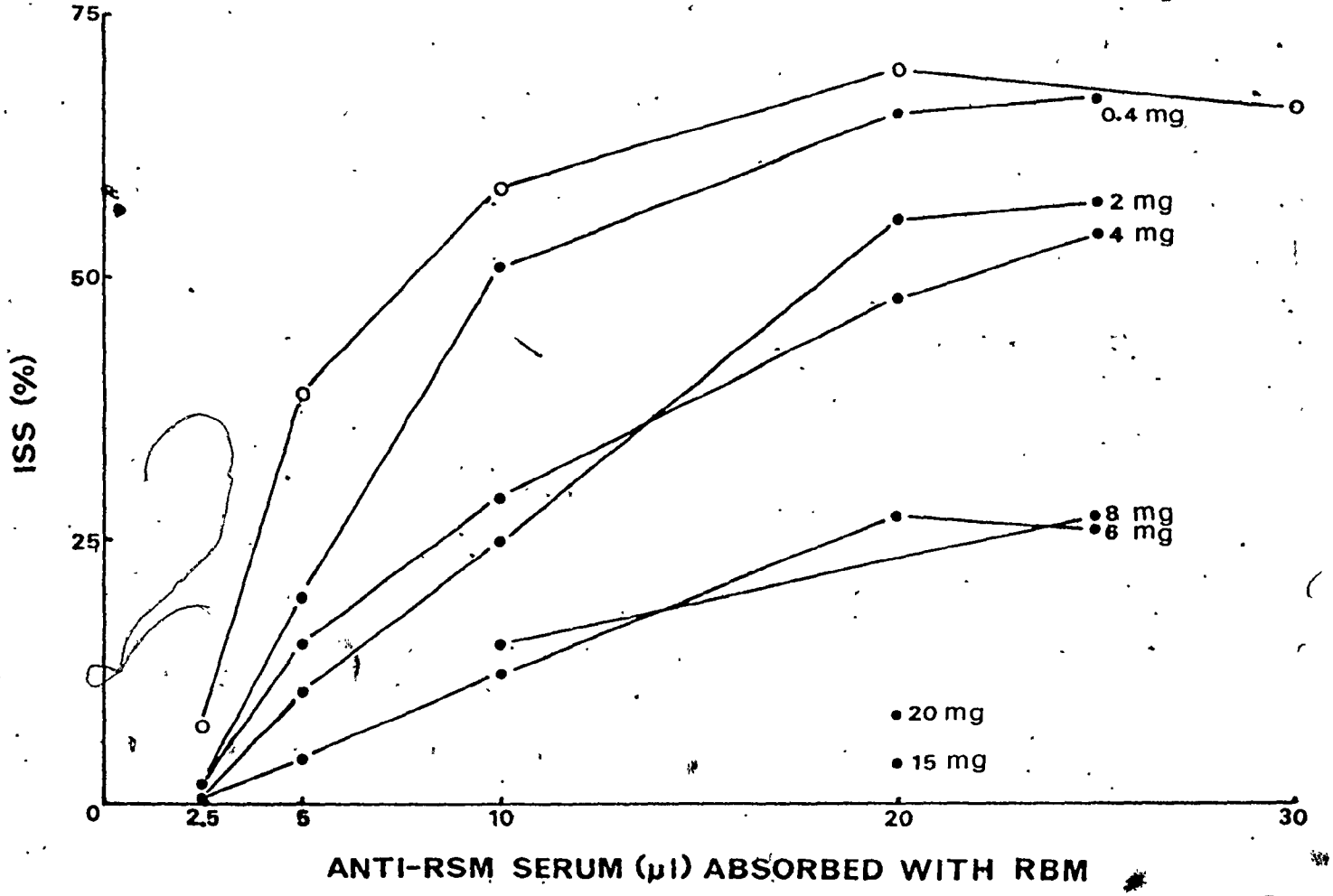

75
50
25
0
ISS (%)
4 mg
2 mg
8 mg
2.5
5
10
20
30
ANTI-RSM SERUM (µl) ABSORBED WITH SYNAPTOSOMES

Figure 6. Decrease in capacity of anti-RSM serum to inhibit synaptosomal swelling after absorption with different amount of RBM protein.

The different amounts of RBM protein used to absorb the serum are indicated alongside each curve (in mg RBM protein/ml serum).

o——— o indicates unabsorbed serum.

•——— • indicates serum absorbed with RBM.


75
50
25
0
ISS (%)
0.4 mg
2 mg
4 mg
8 mg
6 mg
20 mg
15 mg
2.5
5
10
20
30
ANTI-RSM SERUM (µl) ABSORBED WITH RBM

activity. It will be seen from Figure 7 that absorption with 6 and 8 mg of RSM protein removes 80 and 100% of the ISS antibody activity respectively. Absorption with 6 mg of RBM protein removed 60% of the antibody activity; however, 15 mg of RBM protein was needed to remove a further 30% of ISS antibody activity. The residual 5-10% of ISS antibody activity could not be removed by additional RBM. Absorption with synaptosomes can be seen to be about half as effective as RSM.

The sera, that were used for the synaptosomal swelling experiments, were also analysed by the micro-complement fixation technique as described in Materials and Methods. It will be noted from Table 2 that the titres obtained with anti-RSM serum absorbed with various quantities of RSM and RBM are quite similar when the sera are tested with RSM, RBM or synaptosomes. A maximum titre of 320 was obtained with unabsorbed anti-RSM serum with each of the three antigens. The results indicate that absorption of anti-RSM serum with 6 mg of either RBM or RSM effectively decreases the antibody activity to insignificant levels.

The same antisera were also analysed by the immuno-diffusion technique for the presence of precipitating anti-bodies directed against rat brain soluble fraction and Triton extracts of salt washed RSM and RBM. Figure 8 illustrates the immunodiffusion patterns obtained with the various sera. It will be seen that for unabsorbed anti-RSM

Figure 7. Comparison of rat brain synaptosomes, rat brain synaptic membranes and rat brain microsomes for capacity to absorb ISS antibody from anti-RSM serum.

This is a summary of Figure 4, 5 and 6 taking the results obtained from use of 20 µl of absorbed anti-RSM.

o ——— o indicates anti-RSM absorbed with RSM,

• ——— • indicates anti-RSM absorbed with RBM,

+ ——— + indicates anti-RSM absorbed with synaptosomes.

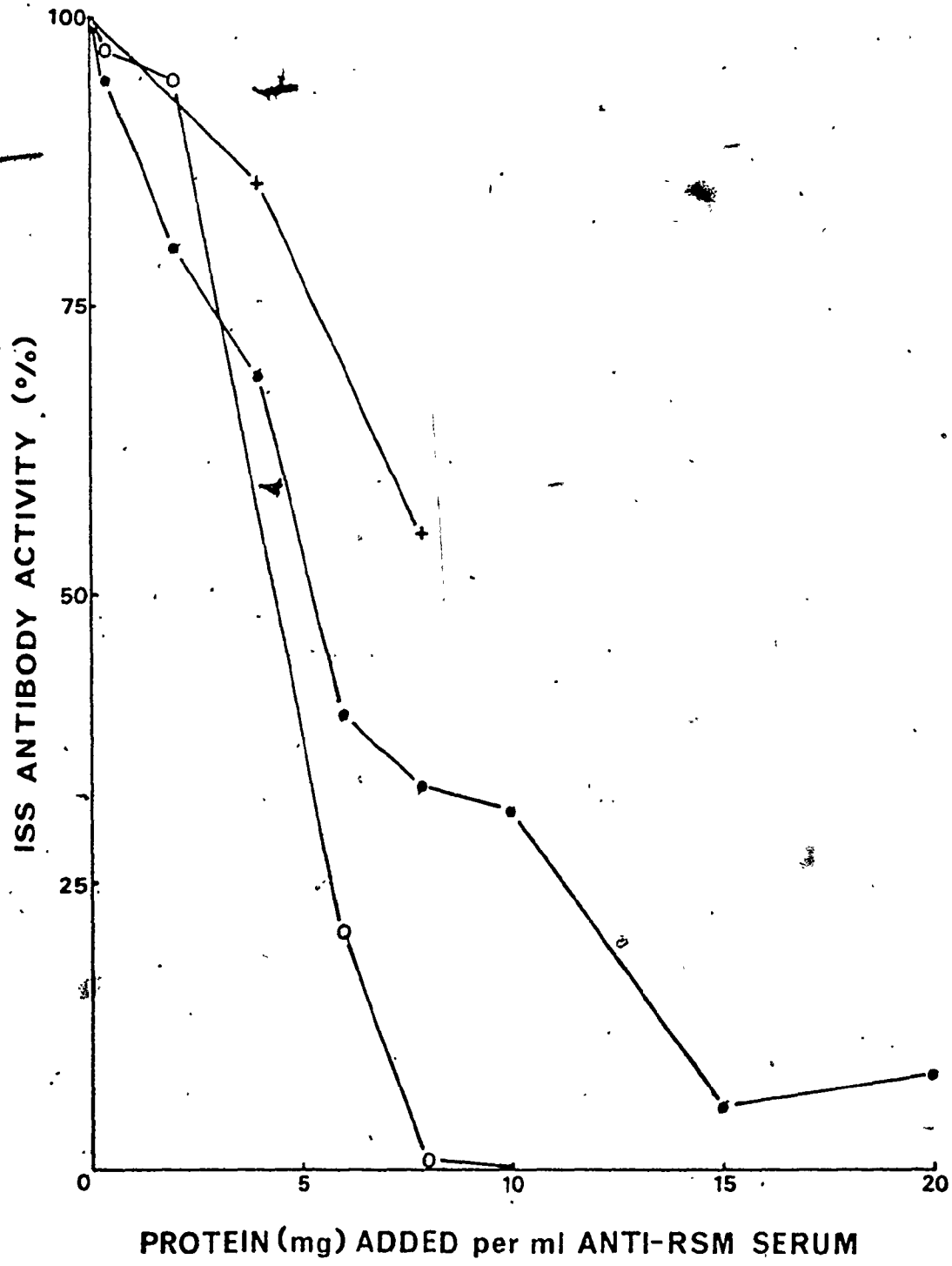

100
75
50
25
0
5
10
15
20
ISS ANTIBODY ACTIVITY (%)
PROTEIN (mg) ADDED per ml ANTI-RSM SERUM

Figure 7A. Comparison of rat brain synaptosomes, rat brain synaptic membranes and rat brain microsomes for capacity to absorb ISS antibody from anti-RSM serum.

This is a summary of Figure 4, 5 and 6 taking the results obtained from use of 10 µl of absorbed anti-RSM.

o——o indicates anti-RSM absorbed with RSM,

•——• indicates anti-RSM absorbed with RBM,

x——x indicates anti-RSM absorbed with synaptosomes.

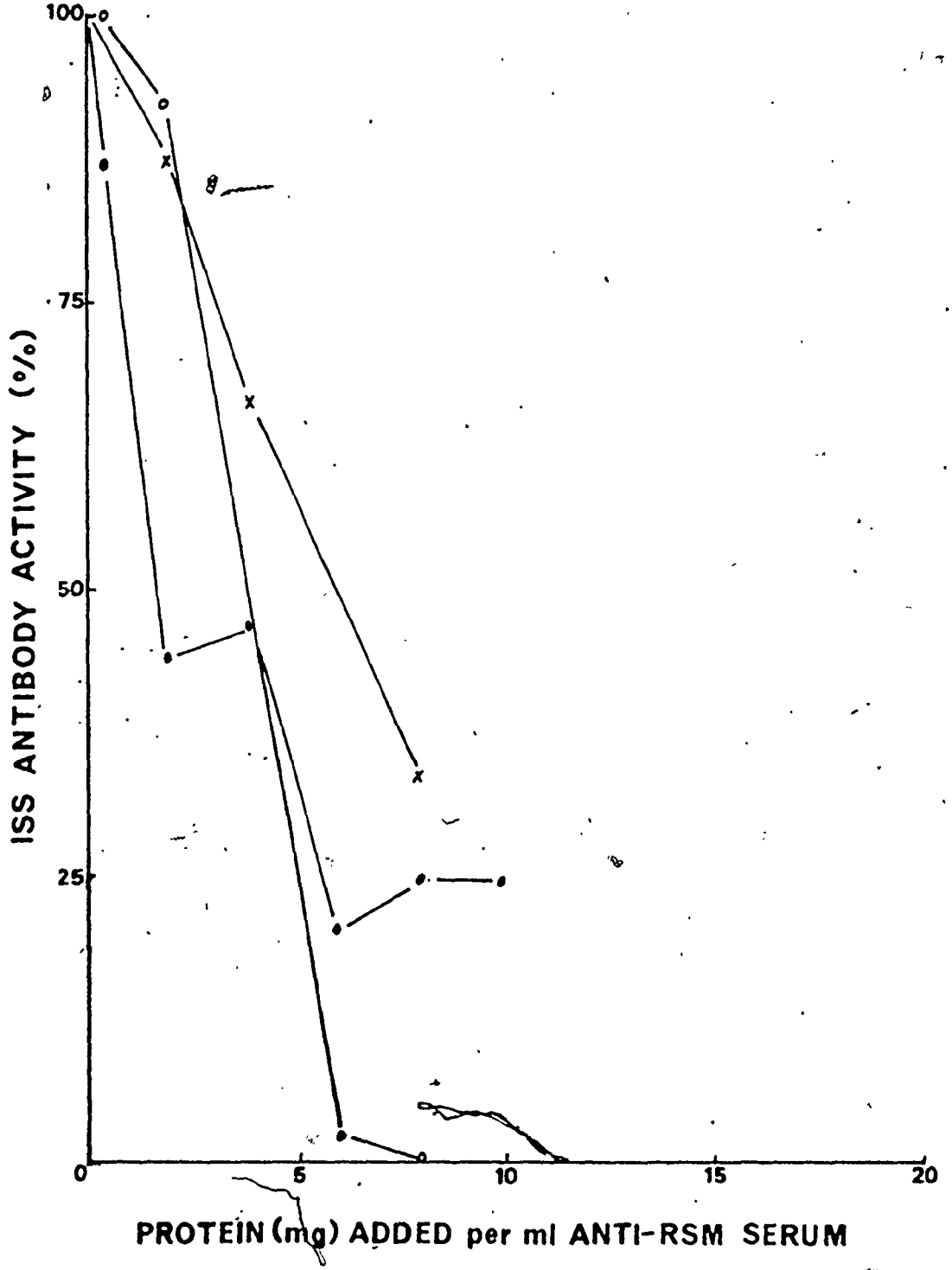

100
75
50
25
0
5
10
15
20
ISS ANTIBODY ACTIVITY (%)
PROTEIN (mg) ADDED per ml ANTI-RSM SERUM

TABLE 2. Comparison of complement fixation titres of anti-RSM serum A before and after absorption with either RSM or RBM(a).

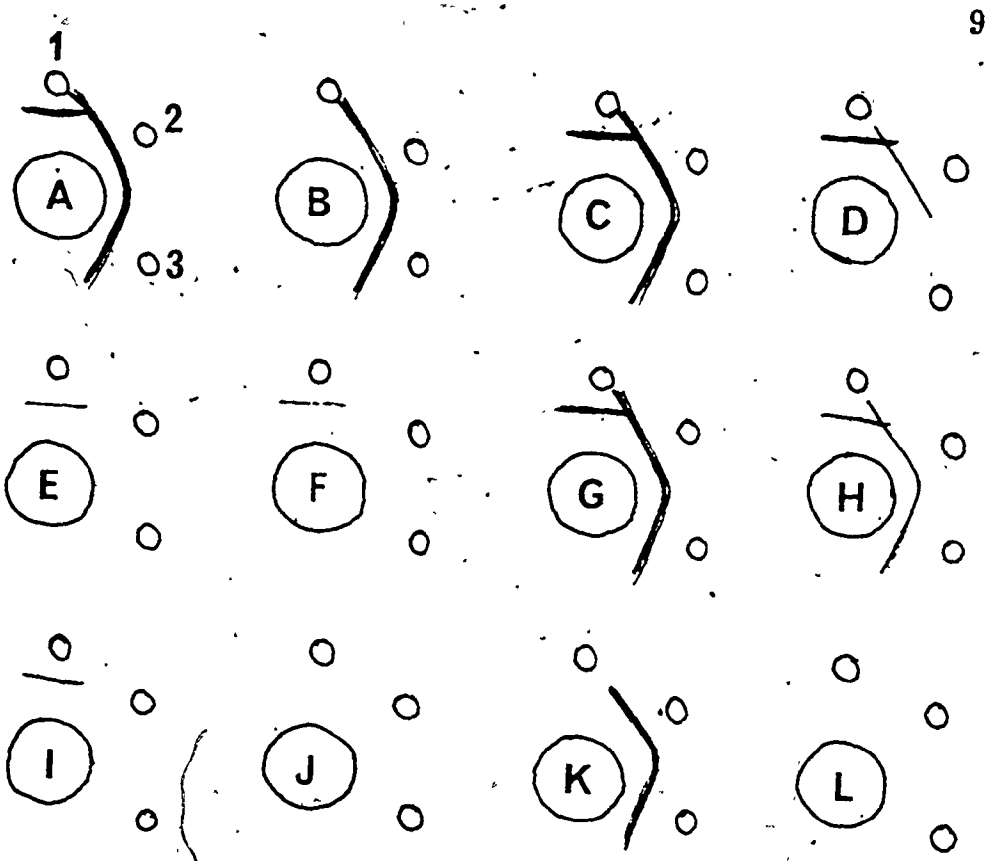
Absorbant	Antigens(b) RSM	RBM	Synaptosomes
-	320(c)	320	320
4 mg RBM	80	20	20
6 mg RBM	2.5	2.5	2.5
8 mg RBM	< 2.5	< 2.5	< 2.5
10 mg RBM	< 2.5	< 2.5	< 2.5
400 µg RSM	320	160	160
2 mg RSM	80	80	80
6 mg RSM	2.5	< 2.5	2.5
8 mg RSM	< 2.5	< 2.5	< 2.5

(a) The results represent the average of two separate series of absorptions.
(b) The sera were tested, for ability to fix complement, in the presence of RSM, RBM or synaptosomes.
(c) The titres are expressed as the reciprocal of the highest dilution of antiserum that gave incomplete hemolysis.

Figure 8. Immunodiffusion analysis of RBE and Triton X-100 (TxO) extracts of RSM and RBM when developed with anti-RSM serum absorbed with RBE, RSM, RBM or synaptosomes.

The peripheral wells contain:
(1) RBE, (2) RSM-TxO, (3) RBM-TxO.
The centre wells contain anti-RSM absorbed with (protein /ml serum):
(A) unabsorbed, (B) 15 mg RBE,
(C) 400 µg RBM, (D) 2 mg RBM,
(E) 4 mg RBM, (F) 6 mg RBM,
(G) 400 µg RSM, (H) 2 mg RSM,
(I) 6 mg RSM, (J) 8 mg RSM,
(K) 4 mg synaptosomes,
(L) 8 mg synaptosomes.

These are drawings of the immunodiffusion patterns that were obtained.


1
2
3
A
B
C
D
E
F
G
H
I
J
K
L

serum (pattern A) two precipitin lines are obtained opposite Triton extracts of RSM and RBM. The two precipitin lines overlap and it is often difficult to distinguish them. A different precipitin line formed opposite the soluble fraction but, after absorption of the serum with soluble fraction this line was no longer detected while the precipitin line formed as usual opposite the Triton extracts of RSM and RBM.

After absorption of the serum with RBM (pattern C to F), there is a progressive decrease in the intensity of the precipitin lines formed opposite the Triton extracts. After absorption with 4 mg of RBM no precipitin lines could be detected opposite the Triton extracts. The intensity of the precipitin line formed opposite rat brain soluble fraction gradually decreases but does not completely disappear.

Serum absorbed with RSM (patterns G to J) yields a pattern similar to the one obtained with RBM as absorbent. There is a progressive decrease in the intensity of the precipitin lines formed opposite the two Triton extracts. Absorption with 2 mg of RSM protein removed one of the lines opposite the Triton extracts, and absorption with 6 mg of RSM eliminated all the antibodies that could be detected by immunodiffusion. The intensity of the precipitin line formed opposite the soluble fraction gradually decreased and was not detectable after absorption with 8 mg of RSM protein.

The intensity of the precipitin lines formed opposite

the Triton extracts was partially decreased after absorption with 4 mg of synaptosomal protein and became imperceptible after absorption with 8 mg of synaptosomal protein. It would seem that synaptosomal protein is more effective than RSM and RBM for removing soluble fraction antibodies since precipitin lines due to this fraction could not be detected after absorption with as little as 4 mg of synaptosomal protein.

The synaptosomal swelling test was used to measure the ISS activity of a pool of anti-RBM serum. Figure 9 is a summary of the results obtained with synaptosomes incubated with 0.2 ml of anti-RBM serum that had been absorbed with increasing amounts of RSM or RBM protein. It will be seen that 40 and 50% of the ISS antibody activity was removed after absorption with 6 mg of RSM and RBM protein respectively.

Absorption of the serum with RBM seems to be more effective in removing ISS antibodies, than is absorption with RSM. The ISS antibody fell to a level of about 30% after 10 mg RBM protein had been used for absorption, and was not decreased by further absorption.

It was decided to investigate the possibility that the microsomal fraction and the mitochondrial fraction might also swell when placed in 0.4M glycerol. Synaptosomal, microsomal and mitochondrial (pellet from Ficoll gradients) preparations were diluted so as to contain 6 mg of protein per ml and incubated with 0.2 ml of normal rabbit serum and complement, and tested for swelling in glycerol. The results in Table 3

Figure 9. Comparison of rat brain synaptic membranes and rat brain microsomes for capacity to absorb ISS antibody from anti-RBM serum.

For the synaptosome swelling assay, 20 µl of serum was used.

o———o indicates anti-RBM serum absorbed with RSM,

•———• indicates anti-RBM serum absorbed with RBM.

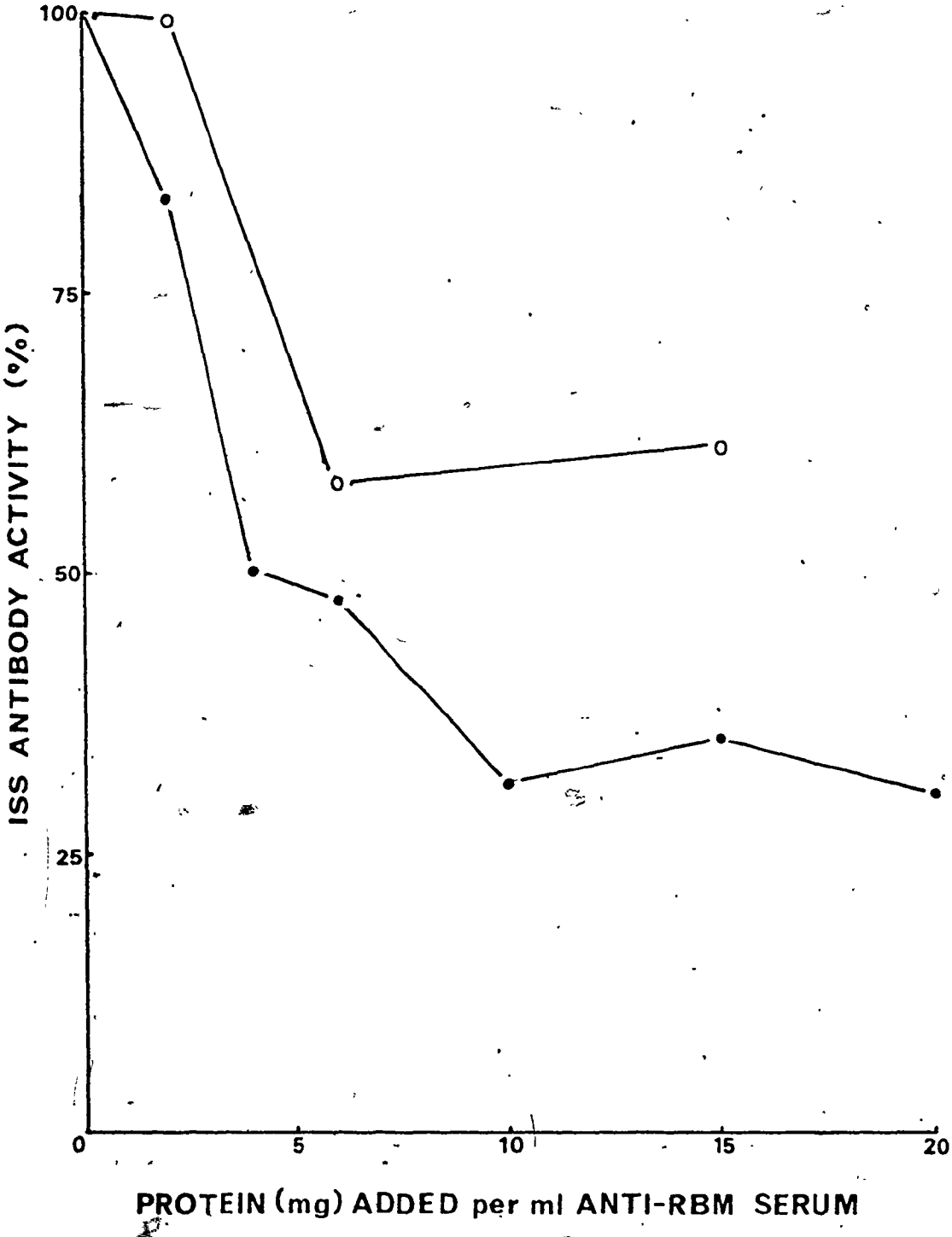

100
75
50
25
0
5
10
15
20
ISS ANTIBODY ACTIVITY (%)
PROTEIN (mg) ADDED per ml ANTI-RBM SERUM

TABLE 3. Swelling of the microsomal and mitochondrial fractions relative to synaptosomal swelling and inhibition of this swelling by anti-RSM serum A before and after absorption with RSM or RBM.

Swelling relative to synaptosomes

	% Swelling
synaptosomal fraction	100%
microsomal fraction	161%
mitochondrial fraction	69%

Fraction subjected to swelling test	% swelling inhibition	% antibody activity
Synaptosomal fraction		
Anti-RSM	67.9	100
Anti-RSM + 8 mg RSM	0.3	0.5
Anti-RSM + 10 mg RBM	21.8	31.4
Microsomal fraction		
Anti-RSM	65.6	100
Anti-RSM + 8 mg RSM	6.3	9.6
Anti-RSM + 10 mg RBM	31.2	47.6
Mitochondrial fraction		
Anti-RSM	80.9	100
Anti-RSM + 8 mg RSM	9.3	11.5
Anti-RSM + 10 mg RBM	9.5	11.7

indicate that the microsomal and mitochondrial fractions swell 161% and 69% respectively as much as the synaptosomal fraction.

The effect of absorbed and unabsorbed anti-RSM serum is summarized in Table 3. It can be seen that the decrease in swelling, due to unabsorbed anti-RSM serum is similar for all three fractions although the inhibition of swelling of the mitochondrial fraction is slightly higher.

After absorption of the serum with 8 mg of RSM protein only 0.5% and 9.6% respectively of the total antibody activity directed towards the synaptosomal and microsomal fractions remained. However, if 10 mg of RBM protein was used as absorbent, 31% and 47% respectively, of the anti-synaptosomal and anti-microsomal activities remained.

The pattern obtained when inhibition of mitochondrial swelling was measured, is different in that absorption of the serum with either RSM or RBM leaves approximately 11% of the antibody activity.

3.3 "WASHING" OF RSM AND RBM

MacPherson, Shek and Pay (1973) have demonstrated that when RBM were sequentially washed with sodium salts, the majority of proteins detected in the salt washings were not antigens and those that were antigens were identified as species-specific proteins that are found in soluble fractions of all rat organs. For the present work, the RSM and RBM were subjected to sequential salt washings procedure. The extrinsic membrane proteins in the saline

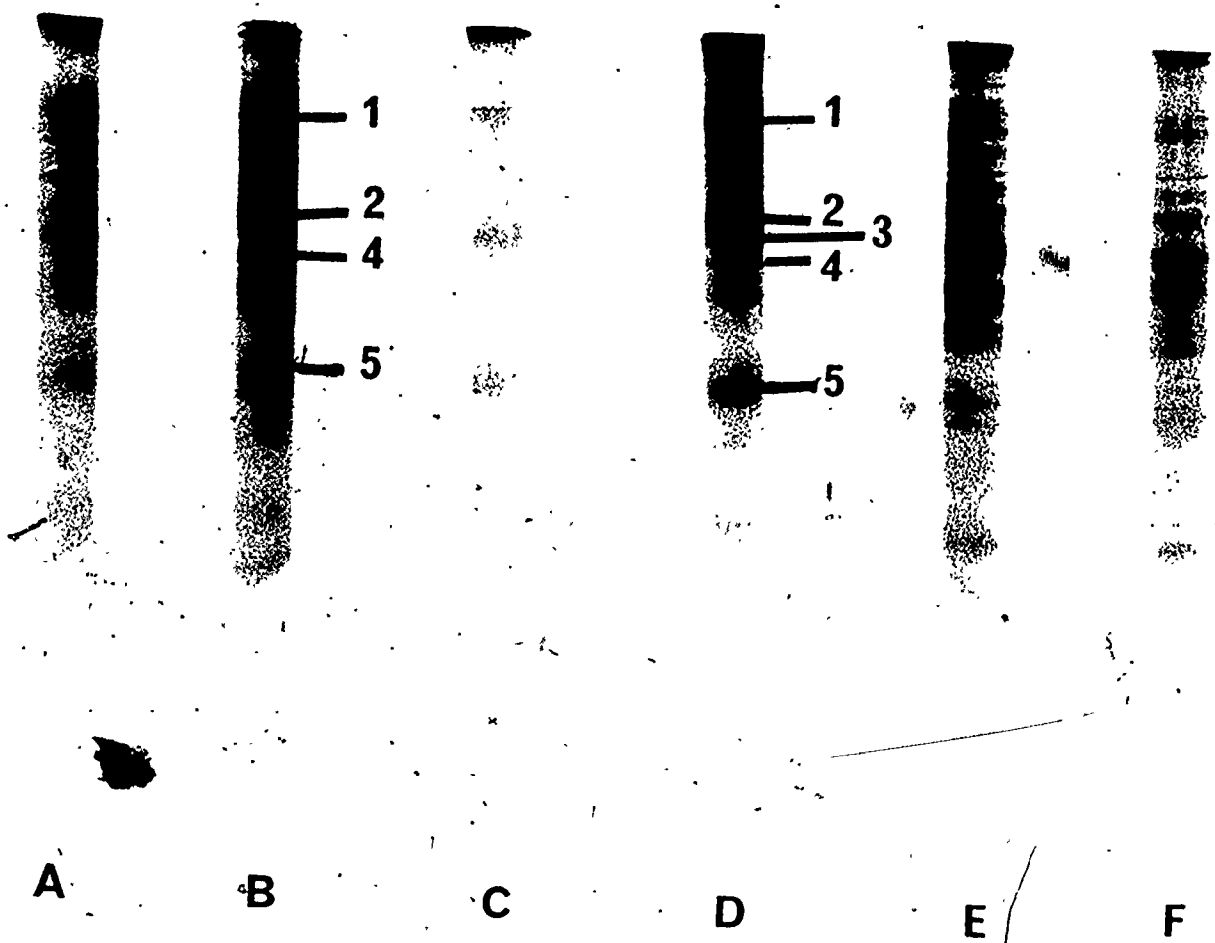
washings of RSM were similar to those in the saline washings of RBM and consisted mainly of soluble fraction components as determined by immunodiffusion tests with anti-RBE sera (as described by MacPherson and Liakopoulou, 1966 and Liakopoulou and MacPherson, 1970).

Figure 10 shows the results of polyacrylamide gel electrophoresis of washed and unwashed RBM and RSM in the presence of SDS, with and without added 2-mercaptoethanol. It will be seen that the patterns obtained in the presence of 2-mercaptoethanol are different from the patterns obtained with SDS alone. For example, it can be seen that clearer, more discrete bands are obtained in gel B as compared to gel A (RBER patterns). Also, the bands labelled 1, 2, and 4 are the major ones (as judged by staining intensity) in gel B, while this does not seem to be the case in gel A. The presence of heavy staining in the first few millimeters at the top of gel A, presumably due to large undissociated complexes, can probably account for the differences observed between gels A and B. Similar differences can be seen when comparing gels C and D, although the presence of 2-mercaptoethanol does not seem to completely dissociate the large complexes of gel D.

When comparing RBER and RSM (gel B and D) it can be seen that the patterns obtained are similar. Quantitative differences can possibly be explained by the presence of undissociated complexes in the RSM pattern. For the same reason, it is difficult to comment on the absence of any

Figure 10. SDS-polyacrylamide gel electrophoretograms of salt washed and unwashed RSM and RBM.

Electrophoresis was carried out in 10% acrylamide gels (0.1% SDS) for 4-5 hr. at 8 mA/gel. The samples were suspended in 1% SDS, with or without added 2-mercaptoethanol (ME) and incubated in a boiling water bath for 3 min. The samples in the gels were : (A) RBM-washed, 80µg; (B) RBM-washed, 80 µg + ME; (C) RSM-washed, 40 µg; (D) RSM-washed, 80µg, + ME; (E) RBM-unwashed, 80µg; (F) RSM-unwashed, 80 µg.
The bands labelled 1, 2, 3, 4, and 5 correspond to molecular weights of 76,000, 51,300, 50,000, 44,000, and 27,000 daltons respectively.


1
2
4
5
1
2
3
4
5
A
B
C
D
E
F

band in the RSM pattern as compared to the RBER pattern. It will be seen, though, that at least one band, labelled number 3, is present in the RSM pattern while it does not seem to be present in the RBER profile.

The patterns obtained with unwashed membranes (gels E and F) do not seem to be qualitatively different from the profiles obtained with washed membranes. Quantitatively, a number of differences can be seen.

Bands 1, 2, 3, 4, and 5 correspond to polypeptides with molecular weights of approximately 76,000, 51,000, 50,000, 44,000, and 27,000 daltons respectively.

3.4 ABSORPTION OF ANTISERA

Anti-RSM serum A and anti-RBM serum A were used for the majority of the immunochemical experiments to be reported. These antisera were raised in rabbits by injection with unwashed membrane preparations. Figure 11 is a representative drawing of the pattern obtained when RBE (soluble fraction of brain) and an RSM TxO extract are allowed to diffuse toward anti-RSM serum A. It will be seen that seven precipitin bands are formed opposite RBE and 4 bands opposite RSM-TxO. Two of the precipitin bands opposite RSM-TxO fused with the band formed opposite RBE.

The antisera used for the immunochemical tests were therefore completely depleted of antibodies directed against the species-specific and organ-specific antigens of the soluble fraction of rat brain by absorption with RBE. When

Figure 11. Immunodiffusion analysis of RBE, normal rat serum and a Triton X-100 (Tx0) extract of RSM developed with anti-RSM IgG before and after absorption with RBE.

The anti-RSM IgG was prepared from anti-RSM serum A.

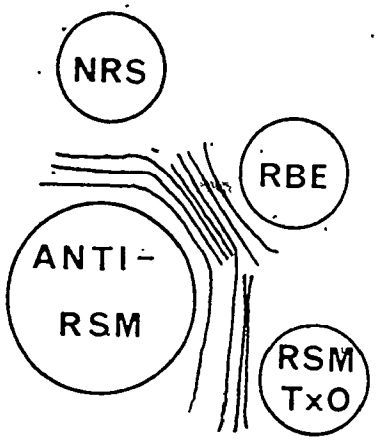
The peripheral wells contained:

(1)normal rat serum, (2)RBE, (3)RSM-Tx0.

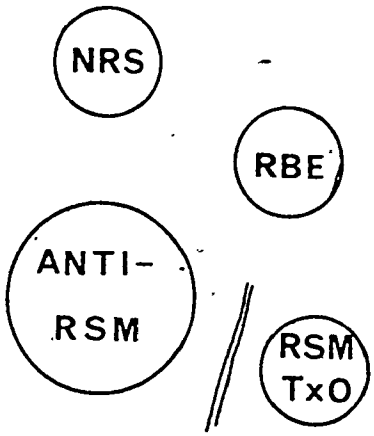
A. The center well contained unabsorbed anti-RSM IgG.

B. The center well contained anti-RSM IgG prepared from anti-RSM serum A absorbed with RBE.

A



B



this is done, the antisera contain antibodies directed only against integral components of RSM and RBER. In Figure 11B, it can be seen that after absorption of the anti-RSM serum with RBE only two precipitin lines were formed opposite RSM TxO extracts. Since the RSM-TxO extract had been prepared from salt-washed RSM, the results indicate that at least some of the antigenic components present in RBE are also intrinsic components of RSM. Similar results were obtained when RBM-TxO extracts and when anti-RBM sera are used.

3.5 EVOLUTION OF TRITON X-100 AND BUTANOL EXTRACTION PROCEDURES

In the early stages of this work, 100 ml of Triton X-100 was used to solubilize membrane components from washed RSM and RBER as described earlier (see Materials and Methods). The extracts were then concentrated by positive pressure dialysis in collodion membranes. About 70% of the membrane protein could be recovered in the extracts. In freshly prepared extracts, two antigens could be detected when the extracts were tested against anti-RSM IgG. These antigens, as detected by immunodiffusion analyses seemed to be labile as greater quantities of Triton extracts were required, after a few weeks, to produce precipitin bands of similar intensity as had been obtained with fresh extracts. Also, the precipitin bands that were formed were diffuse so that it was often difficult to visualize the two antigens (Figure 11B).

The components responsible for the outer and inner precipitin bands were designated X1 and X2 respectively.

The Triton extraction procedure was then modified by incubating the membranes with a small volume of 2% Triton X-100 followed by extraction of the pellet with 100 ml of 1% Triton X-100 (see Chapter 2, section 2.2.5.1, Method 2). The two extracts, TxA and TxB were then concentrated by flash evaporation. The yield of antigens X1 and X2, obtained by this method was higher than obtained from the extraction by Method 1. Method 2 is also a much faster procedure as the extracts could be tested on the same day.

Figures 12A and 12B are photographs of the same slide taken after 36 and 48 hours of development. These are included to illustrate the different rates of development of the precipitin bands formed by X1 and X2. It will be seen from Figure 12A that two distinct precipitin lines appeared opposite the butanol extract of RBER whereas one distinct and one faint line formed opposite the TxA extract of RBER. By the time that the faint outer precipitin band opposite RBER-TxA could be clearly seen, the inner band had broadened and faded (Figure 12B). Two precipitin bands, which generally did not separate clearly also formed opposite the RSM-TxA extract. In Figure 12B, a very faint outer precipitin band is also seen opposite the RSM-TxB extract. When Triton extracts of RBER and RSM are placed in neighbouring wells, the precipitin bands opposite all four Triton extracts fused, indicating that the two antigens were identical in RSM and RBER. A third precipitin

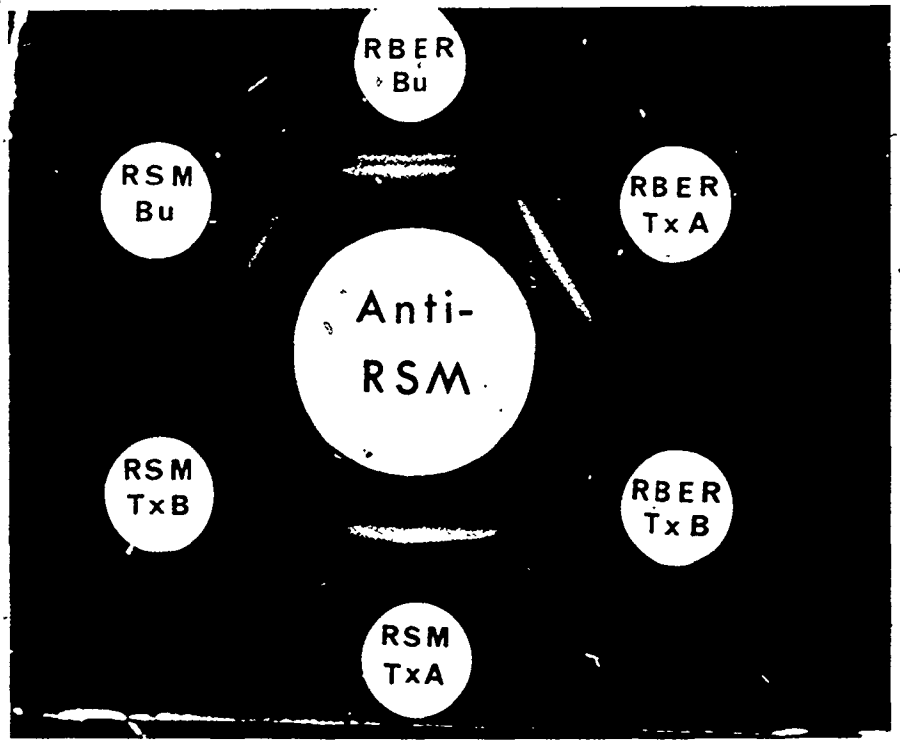
FIGURE 12. Immunodiffusion analysis of Triton X-100 (TxA and TxB) and butanol (water phase) extracts of RBER and RSM developed with anti-RSM IgG.

The slides were coated with 1.2% agarose containing 0.05M barbital buffer, pH 8.6, and 1% Triton X-100. The anti-RSM IgG was prepared from anti-RSM serum A absorbed with RBE. Protein concentrations in the peripheral wells were as follows: RBER-Bu, 13.1 mg/ml; RBER-TxA, 21.2 mg/ml; RBER-TxB, 2.1 mg/ml; RSM-TxA, 10 mg/ml; RSM-TxB, 1.5 mg/ml; RSM-Bu, 3.5 mg/ml.

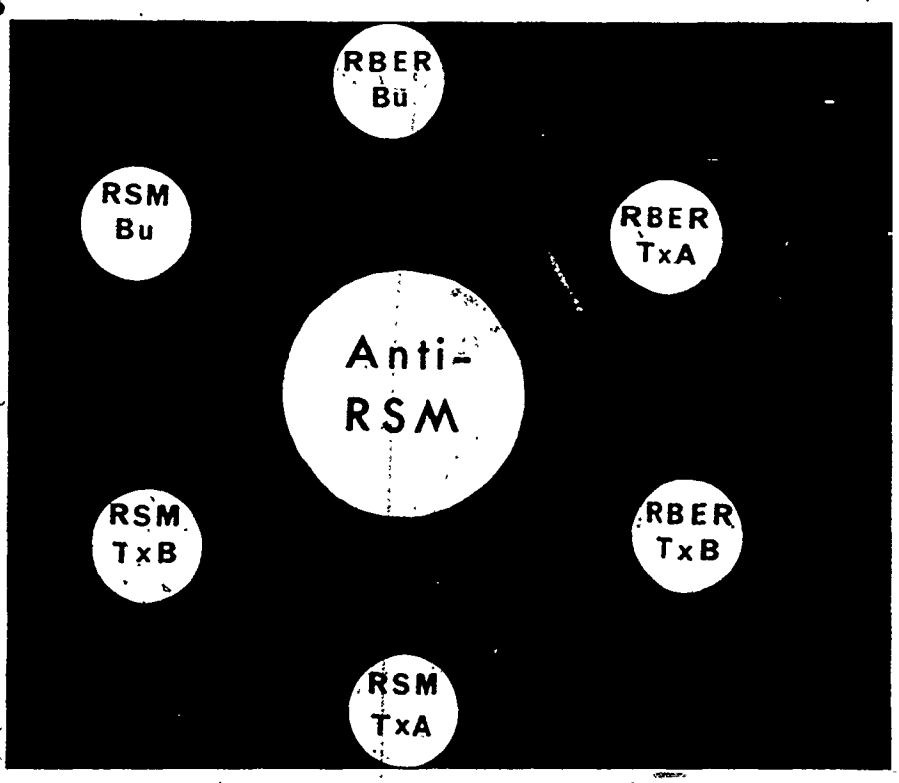
A. Photograph taken after 36 hr.

B. Photograph taken after 48 hr.

A


RBER Bu
RSM Bu
RBER TxA
Anti-RSM
RSM TxB
RBER TxB
RSM TxA

B


RBER Bu
RSM Bu
RBER TxA
Anti-RSM
RSM TxB
RBER TxB
RSM TxA

band can also be seen opposite the RBER-TxA extract.

Solutions obtained by re-extraction of RBER with Triton (TxB) or by the treatment of RSM with butanol formed a single precipitin line (due to antigen X2) that fused with the inner band formed by the other four extracts. Precipitin lines due to antigen X1 could sometimes be seen (very weak) opposite RBER-TxB and RSM-Bu.

These results indicated that antigens X1 and X2 could be sequentially extracted from RSM and RBER. By trial and error, the Triton extraction procedure was modified so as to obtain Triton extracts containing antigen X2 and not X1 (see section 2.2.5.1, Method 3). The volume of Triton X-100 used for the initial extraction was increased and this treatment was repeated once to yield Tx1a and Tx1b extracts. A final extraction with 100 ml of 1% Triton X-100 then gave a Tx2 extract which contains only antigen X2.

The butanol extraction was also modified in that Triton X-100 was added to the aqueous extracts before concentration. Initially, the butanol extracts had been concentrated in collodion membranes (without Triton X-100). The antigenic activities were preserved for several months when the concentrated extracts contained 0.5% Triton X-100 and were stored at 4°C.

Figure 13A is a photograph of immunodiffusion results obtained with the modified Triton and butanol extracts. Aprotinin (5%) was added to the agarose. It will be seen that two distinct precipitin lines formed opposite the Tx1a

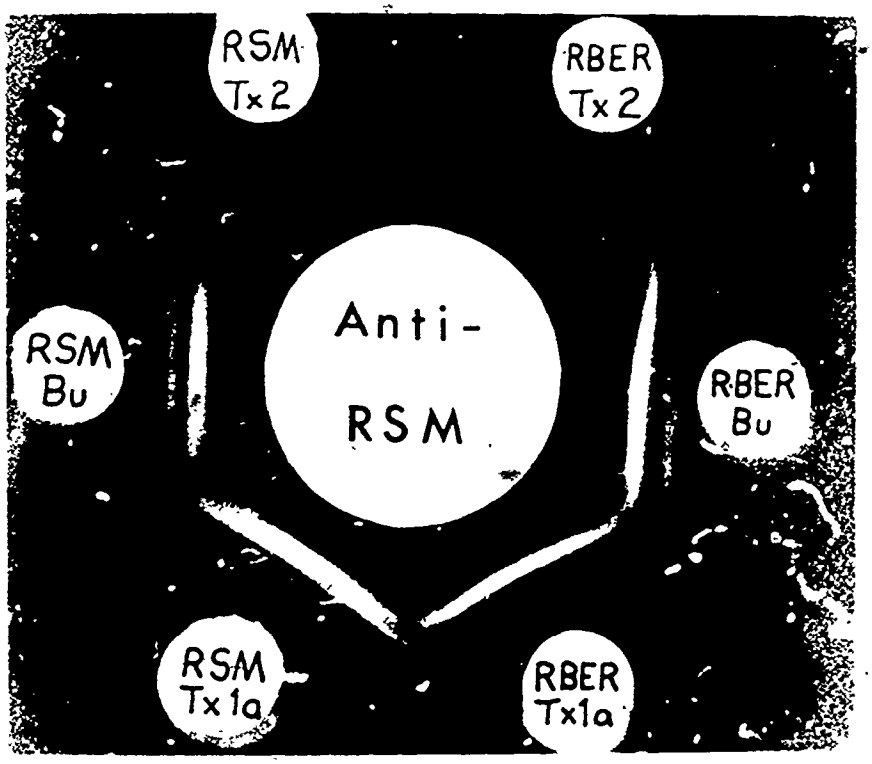
FIGURE 13. Immunodiffusion analyses of Triton X-100 and butanol (water phase) extracts of RBER and RSM developed with anti-RSM IgG and anti-RBM IgG.

The slides were coated with 1.2% agarose containing 0.5M barbital buffer, pH 8.6, 1.0% Triton X-100 and 5% aprotinin. The protein concentrations in the peripheral wells were as follows:
RBER-Tx2, 4.9 mg/ml; RBER-Bu, 16.7 mg/ml; RBER-Txla, 11.0 mg/ml; RSM-Txla, 11.9 mg/ml; RSM-Bu, 10.5 mg/ml; RSM-Tx2, 3.8 mg/ml.

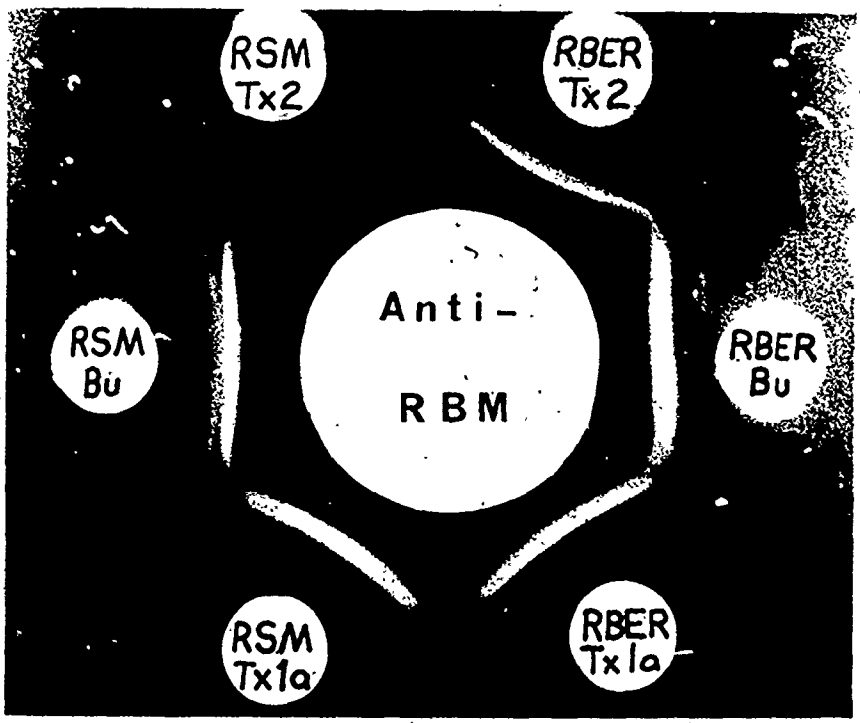
A. The anti-RSM IgG was prepared from anti-RSM serum A absorbed with RBE.

B. The anti-RBM IgG was prepared from anti-RBM serum A absorbed with RBE.

A


RSM Tx2
RBER Tx2
Anti-RSM
RSM Bu
RBER Bu
RSM Tx1a
RBER Tx1a

B


RSM Tx2
RBER Tx2
Anti-RBM
RSM Bu
RBER Bu
RSM Tx1a
RBER Tx1a

and the butanol extracts of RSM and RBER when the extracts were allowed to diffuse toward anti-RSM IgG (prepared from serum A). The use of aprotinin has allowed the precipitin bands due to X1 and X2 to be photographed at the same time. The aprotinin appears to facilitate the diffusion of certain antigens from the peripheral wells.

The two precipitin bands opposite the Txla extracts of RSM and RBER fused, indicating that X1 and X2 were identical in RSM and RBER. In contrast to Figure 12, the butanol extract of RSM can be seen to form two precipitin bands. The use of aprotinin and the presence of Triton X-100 to stabilize the antigens have enabled the detection of X1.

Tx2 extracts of RSM and RBER formed a single precipitin line that fused with the inner band formed by the Txla and butanol extracts of both membranes. Therefore, the Tx2 extracts contain X2 and not X1.

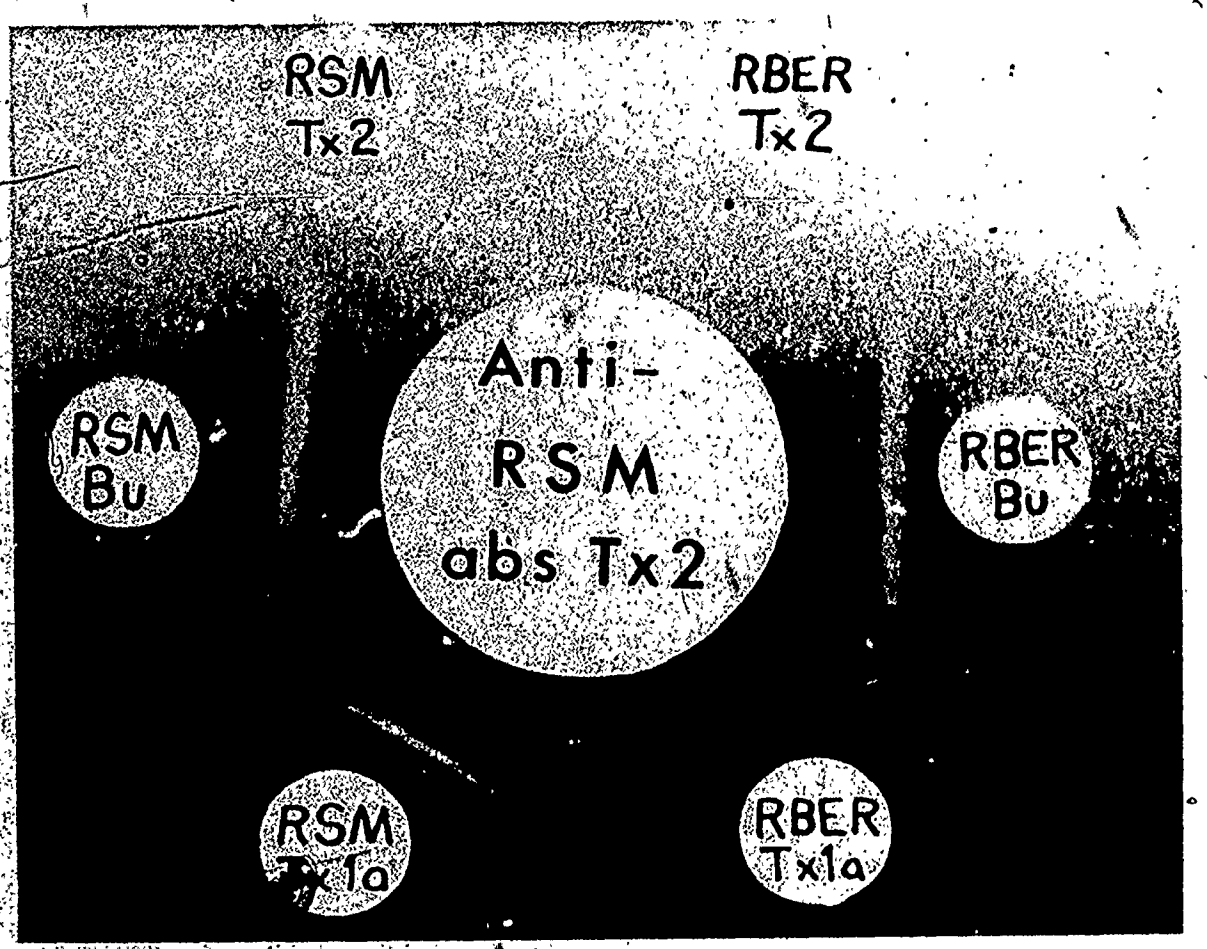
If the anti-RSM IgG in the center well was replaced by anti-RBM IgG A the same pattern appeared (Figure 13B) indicating that the antibodies formed in response to immunization with RBM were identical to the anti-X1 and anti-X2 antibodies formed by immunization with RSM.

Figure 14 shows the results of an immunodiffusion analysis with anti-RSM absorbed with an RSM-Tx2 extract that contained only antigen X2. It will be seen that only one line formed opposite aqueous extracts of butanol treated RSM and RBER and opposite Txla extracts of RSM and RBER showing that only anti-X1 antibody remained in the anti-RSM IgG.

FIGURE 14. Immunodiffusion analysis of Triton X-100 and butanol (water phase) extracts of RBER and RSM developed with anti-RSM IgG absorbed with RBE and RSM-Tx2.

The slides were coated with 1.2% agarose containing 0.05M barbital buffer, pH 8.6, 1.0% Triton X-100 and 5% aprotinin.

The protein concentrations in the peripheral wells were the same as in Figure 13. The anti-RSM IgG was prepared from anti-RSM serum A.


RSM
Tx2
RBER
Tx2
RSM
Bu
Anti-
RSM
abs Tx2
RBER
Bu
RSM
Tx1a
RBER
Tx1a

In Figure 15A, anti-RSM IgG and anti-RBER serum B are compared by immunodiffusion with an RSM-Bu extract. It will be seen that two precipitin lines formed opposite the anti-RSM IgG while only one band formed opposite anti-RBER serum B, this band being due to antigen X2, indicating that anti-RBER serum B contains mainly anti-X2 antibodies. Figure 15B is an immunodiffusion analysis of anti-RBER serum B developed with Triton X-100 extracts and aqueous extracts of butanol treated membranes. It will be seen that only one continuous line is present which was formed by antigen X2.

3.6 IMMUNOELECTROPHORESIS OF TRITON AND BUTANOL EXTRACTS

Immunoelectrophoretic patterns of Triton X-100 extracts of RSM and RBER were developed with the anti-RSM IgG and are shown in Figure 16A. The main feature of these immunoelectrophoretic profiles is a single wide precipitin arc having a midpoint somewhere to the cathodal side of the origin. Thus, the immunoelectrophoretic mobility in agarose at pH 8.6 of the major antigen (X2) in Triton X-100 extracts of RSM and RBER is similar to that of a serum γ-globulin run under the same conditions. There is also a second precipitin arc due to X1. It will be noted that the patterns of Triton X-100 extracts of RSM and RBER are indistinguishable.

FIGURE 15A. Immunodiffusion analysis of a butanol extract (water phase) of RSM developed with anti-RSM IgG and anti-RBER serum B.

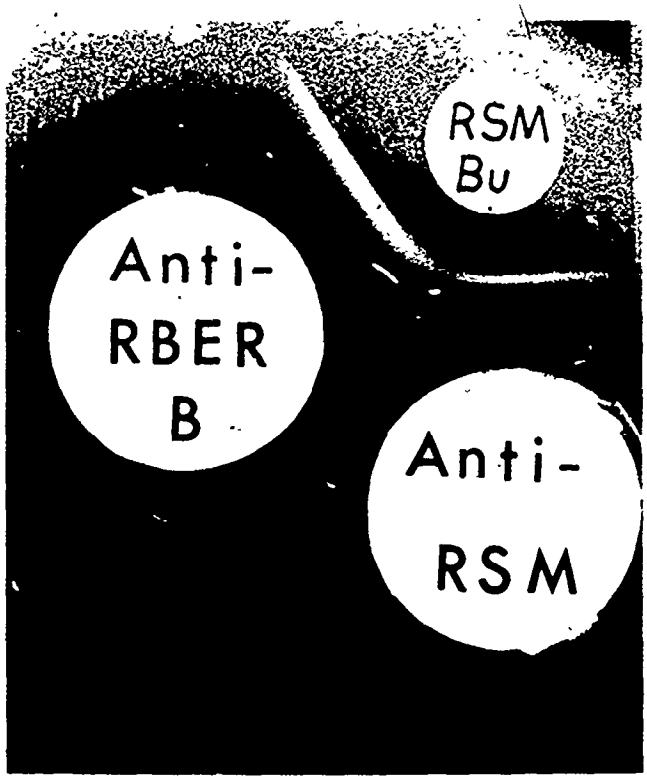
B. Immunodiffusion analysis of Triton X-100 and butanol (water phase) extracts of RBER and RSM developed with anti-RBER serum B.

The slides were coated with 1.2% agarose containing 0.05M barbital buffer, pH 8.6, 1.0% Triton X-100 and 5% aprotinin.

The anti-RSM IgG was prepared from anti-RSM serum A absorbed with RBE. The anti-RBER serum B was raised by immunization of rabbits with "salt washed" RBER.

The protein concentrations in the peripheral wells were the same as in Figure 13.

A


RSM
Bu
Anti-
RBER
B
Anti-
RSM

B

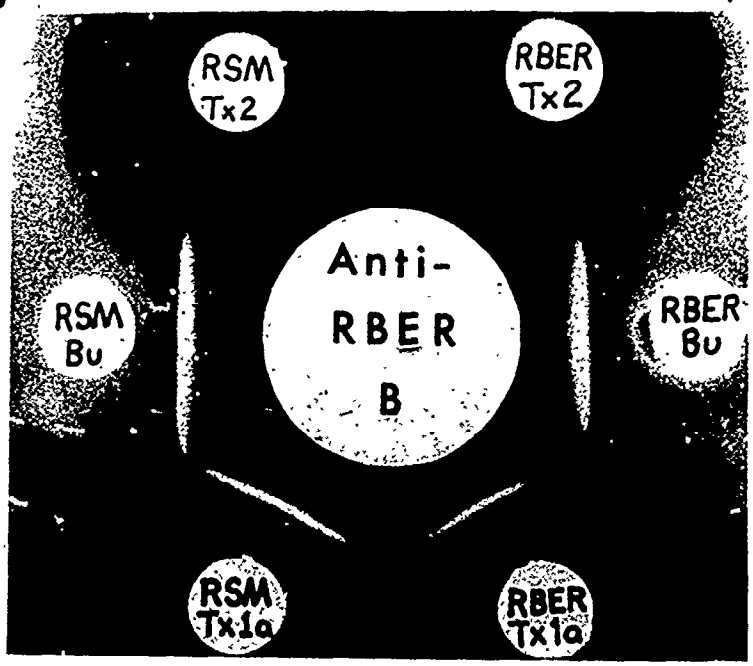
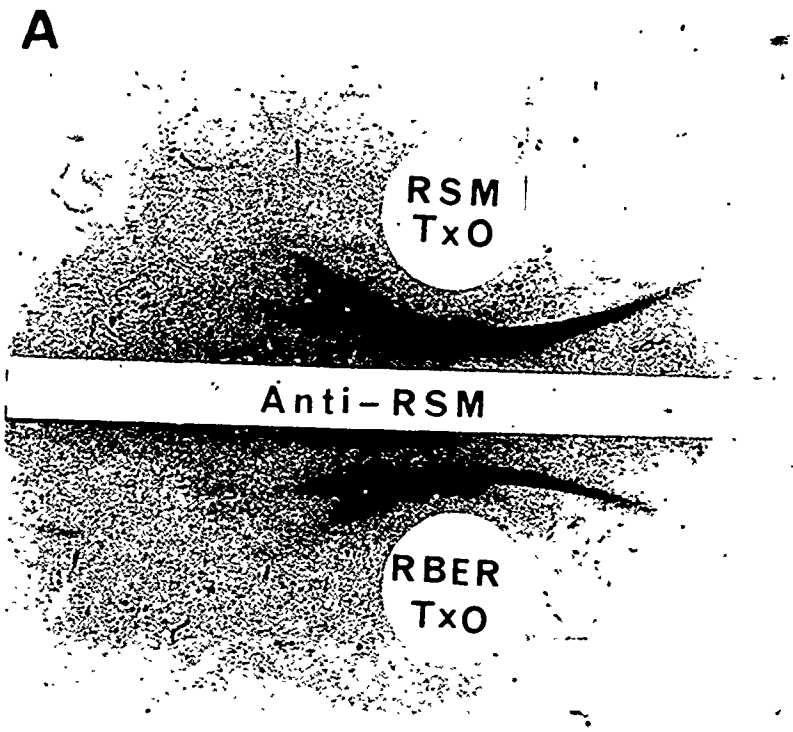

RSM
Tx2
RBER
Tx2
Anti-
RBER
B
RSM
Bu
RBER
Bu
RSM
Tx1a
RBER
Tx1a

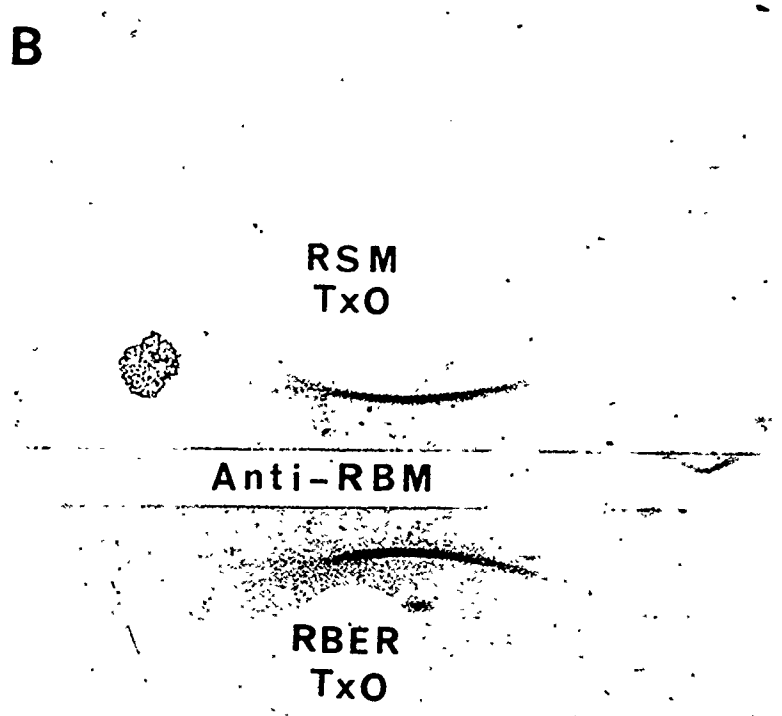
Figure 16. Immunoelectrophoretic analyses of Triton X-100 extracts of RBER and RSM developed with anti-RSM IgG and anti-RBM IgG.

The slides were coated with 1.2% agarose containing 0.05M barbital buffer, pH 8.6, and 1% Triton X-100. Electrophoresis was carried out for 45 min at 15 mA per slide. The cathode is on the right.

A. The anti-RSM IgG was prepared from anti-RSM serum A absorbed with RBE.

B. The anti-RBM IgG was prepared from anti-RBM serum A absorbed with RBE.


A
RSM
TxO
Anti-RSM
RBER
TxO


B
RSM
TxO
Anti-RBM
RBER
TxO

Similar immunoelectrophoretic patterns of Triton X-100 extracts of RSM and RBER were obtained when the extracts were allowed to develop with the anti-RBM IgG A (Figure 16B).

The immunoelectrophoretic profile of butanol extracts of RSM shown in Figure 17A is of great interest and usually consisted of a single continuous line in the form of a double (batwing) arc. Such a pattern indicates that two molecular forms of the major integral membrane antigen are present in butanol extracts, one form being substantially more basic than the molecular form extracted by Triton X-100. Moreover, the two arcs in the pattern of RSM are of similar density indicating that the two forms of X2 are present in roughly equal amounts. In contrast, the electrophoretic pattern of the butanol extracts of RBER show that only a small amount of the more basic form of the X2 is present in RBER. A similar pattern is obtained with anti-RBM IgG A (Figure 17B).

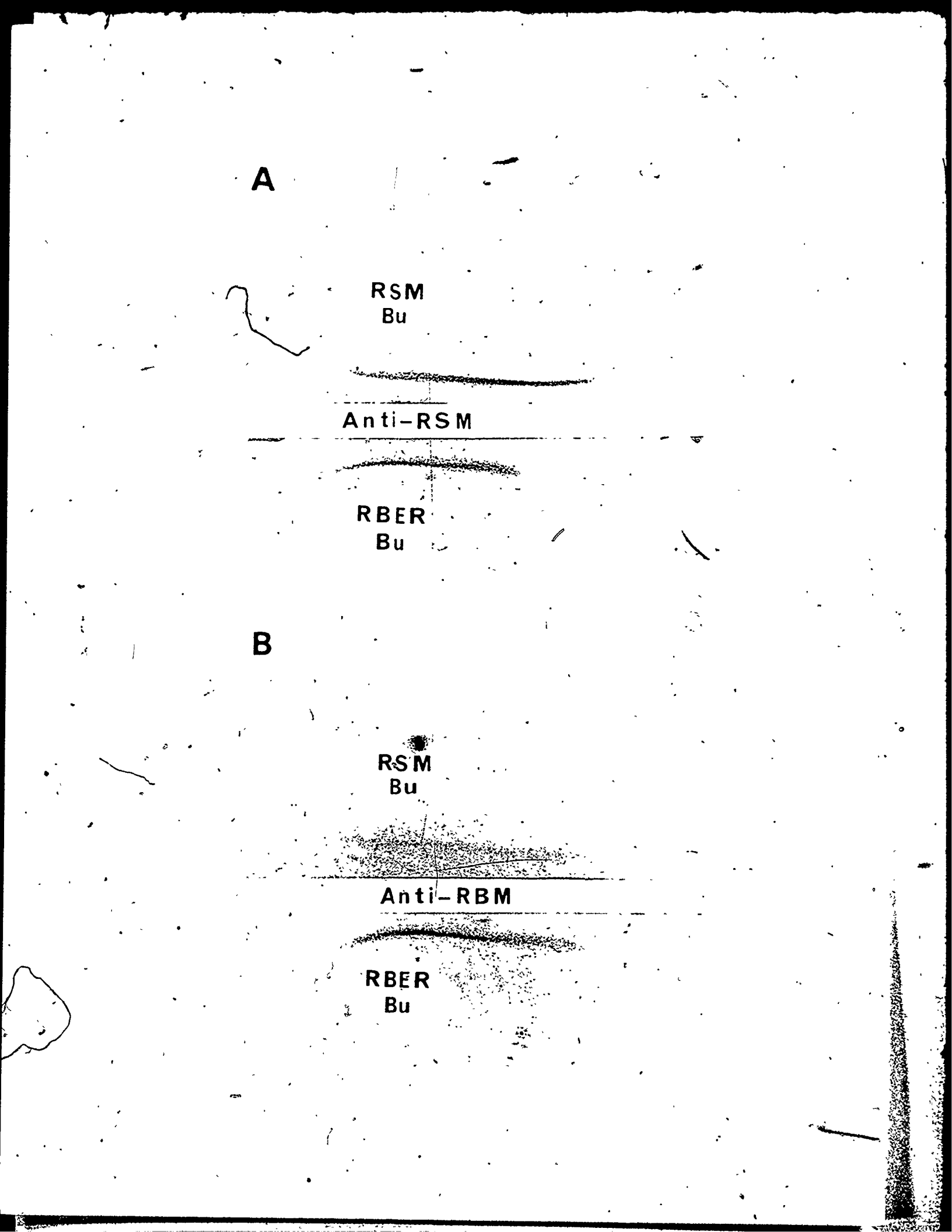
When Triton X-100 was used to preserve the antigenic activity of the butanol extracts, the immunoelectrophoretic patterns obtained with the modified butanol extracts were different as the basic form of X2 could no longer be detected. Precipitin arcs, due to antigen X1, could be seen. In effect, the patterns resembled the profiles obtained with Triton extracts. It is possible that the more basic form of X2, present in the butanol extracts, has been masked by the formation of Triton micelles. This raises the possibility that the more basic form of X2 is actually present (masked) in the Triton X-100 extracts themselves.

Figure 17. Immunoelectrophoretic analyses of butanol extracts (water phase) of RBER and RSM developed with anti-RSM IgG and anti-RBM IgG.

The slides were coated with 1.2% agarose containing 0.05M barbital buffer, pH 8.6, and 1% Triton X-100. Electrophoresis was carried out for 45 min at 15 mA per slide. The cathode is on the right.

A. The anti-RSM IgG was prepared from anti-RSM serum A absorbed with RBE.

B. The anti-RBM IgG was prepared from anti-RBM serum A absorbed with RBE.


A
RSM
Bu
Anti-RSM
RBER
Bu
B
RSM
Bu
Anti-RBM
RBER
Bu

3.7 MOLECULAR WEIGHT ESTIMATION OF X2

Butanol extracts of RBER were eluted from a calibrated column of Sepharose 6B, with 0.05M Phosphate buffer, pH 7.4, containing 0.15M NaCl. Ten ml fractions were concentrated by flash evaporation to about 1 ml and tested for the presence of antigen X2, in immunodiffusion plates using an anti-RSM IgG A. Antigen X2 was detected at an elution volume corresponding to 260,000 daltons. When the Sepharose 6B was equilibrated with butanol saturated phosphate buffer, X2 was eluted at a volume corresponding to 190,000 daltons.

Similarly, RSM-TxO extracts were eluted from a calibrated Sepharose 6B column equilibrated with 0.05M phosphate buffer, pH 7.4, containing 0.14M NaCl and 1% Triton X-100. Antigen X2, was eluted at a volume corresponding to a molecular size of 180,000 daltons.

The problem with using Triton extracts for gel filtration is that most of the proteins, including antigen X2, are eluted together with the major portion of Triton X-100. It is possible that antigen X2 is present as an aggregate but since most of the proteins, present in the extract, are eluted together, it is possible that all these proteins form part of Triton X-100 micelles. This same pattern is obtained when Sephadex G-100 or G-200 are used.

If the gel and the Triton X-100 extracts are equilibrated with SDS, the elution patterns are improved. Thus, RSM-TxO extracts were eluted from a calibrated Bio-gel P-150 column equilibrated with 0.1% SDS. Antigen X2 was detected in an

elution volume corresponding to 28,000 daltons.

Attempts were made to estimate the size of X2 by comparing the migration of a butanol extract of RBER in polyacrylamide gels with proteins of known molecular weight. A Butanol extract of RBER, equilibrated with dialysis buffer containing 2% SDS, was placed on gels containing 10% polyacrylamide and 0.1% SDS. After electrophoresis, the gels were embedded in agarose (containing 1% Triton X-100) and a trough, adjacent to the gels, was filled with anti-RBM IgG. The position of X2 was determined by measuring the mid-point of the precipitin arc and by comparing this distance to the migration in stained gels of proteins of known molecular weights. The midpoint of the arc was about 3 cm from the top of the gel. This corresponded to a molecular size of approximately 30,000 daltons.

An ammonium sulfate precipitate (9% saturation) obtained from an RBER-TxO extract was studied in the same way. The migration of antigen X2 corresponded to a molecular size of approximately 22,000 daltons.

Triton X-100 was removed from RBER-Tx1a and Tx2 extracts by lyophilizing the extracts and washing sequentially with butanol and acetone. The precipitate remaining after this procedure is soluble in warm water. These extracts were equilibrated with phosphate buffer containing 0.1% SDS, allowed to separate on 10% polyacrylamide gels and embedded in agarose as above. The midpoint of the precipitin arcs, correspond to molecular sizes of approximately 36,000 and

32,000 daltons for the Txla and Tx2 extracts respectively. Figure 18 compares the migration of antigen X2 to the electrophoretic profiles (stained) obtained with RBER Txla and Tx2 extracts. It should be mentioned that the migration of X2 (in cm.) had to be corrected, since stained gels swell (increase in length) by about 11% when placed in the destaining solution.

3.8 PURIFICATION OF ANTIGEN X2

Attempts were made to purify antigen X2. Sephadex G-75 equilibrated with 0.5% SDS, was used for the gel filtration of RBER-Txla extracts from which Triton X-100 had been removed by lyophilization and washing with butanol and acetone. The elution pattern obtained is shown in Figure 19. It will be seen that there was poor separation of the protein components present in the extracts. The fractions obtained were pooled in 10 ml lots and the protein precipitated with 9 volumes of acetone. This treatment removes most of the SDS. The precipitates were each solubilized in 0.5 ml of water and used for immunodiffusion tests. If the fractions were concentrated by flash evaporation, the SDS inhibited formation of precipitin bands. After analysis of the fractions by immunodiffusion, it was seen that antigen X2 separated from antigen X1. By analysis of the fractions on SDS-polyacrylamide gels, it could be seen that the last fraction which contained X2 was relatively uncontaminated with other proteins as compared to the initial RBER-Txla extract.

Figure 18. Molecular weight estimation of X2 by SDS-polyacrylamide gel electrophoresis followed by immunodiffusion in agarose.

A and B represent RBER-Tx1A electrophoretograms (A, after staining; B, embedded in agarose)
D and E represent RBER-Tx2 electrophoretograms (D, embedded in agarose; E, after staining).
C indicates a trough cut in the agarose and filled with anti-RSM IgG.
The arrows indicate precipitin arcs formed by X2. The centre of the precipitin arcs formed by the Tx1a and the Tx2 extracts correspond to molecular weights of 36,000 and 32,000 daltons respectively.

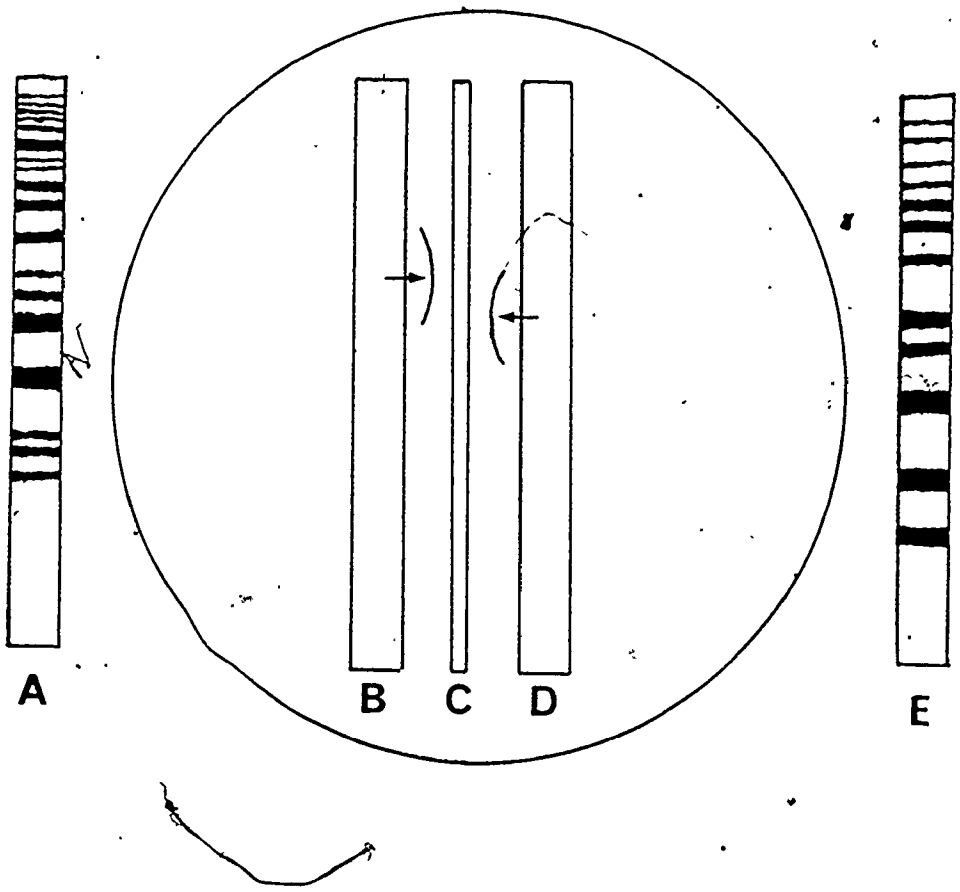
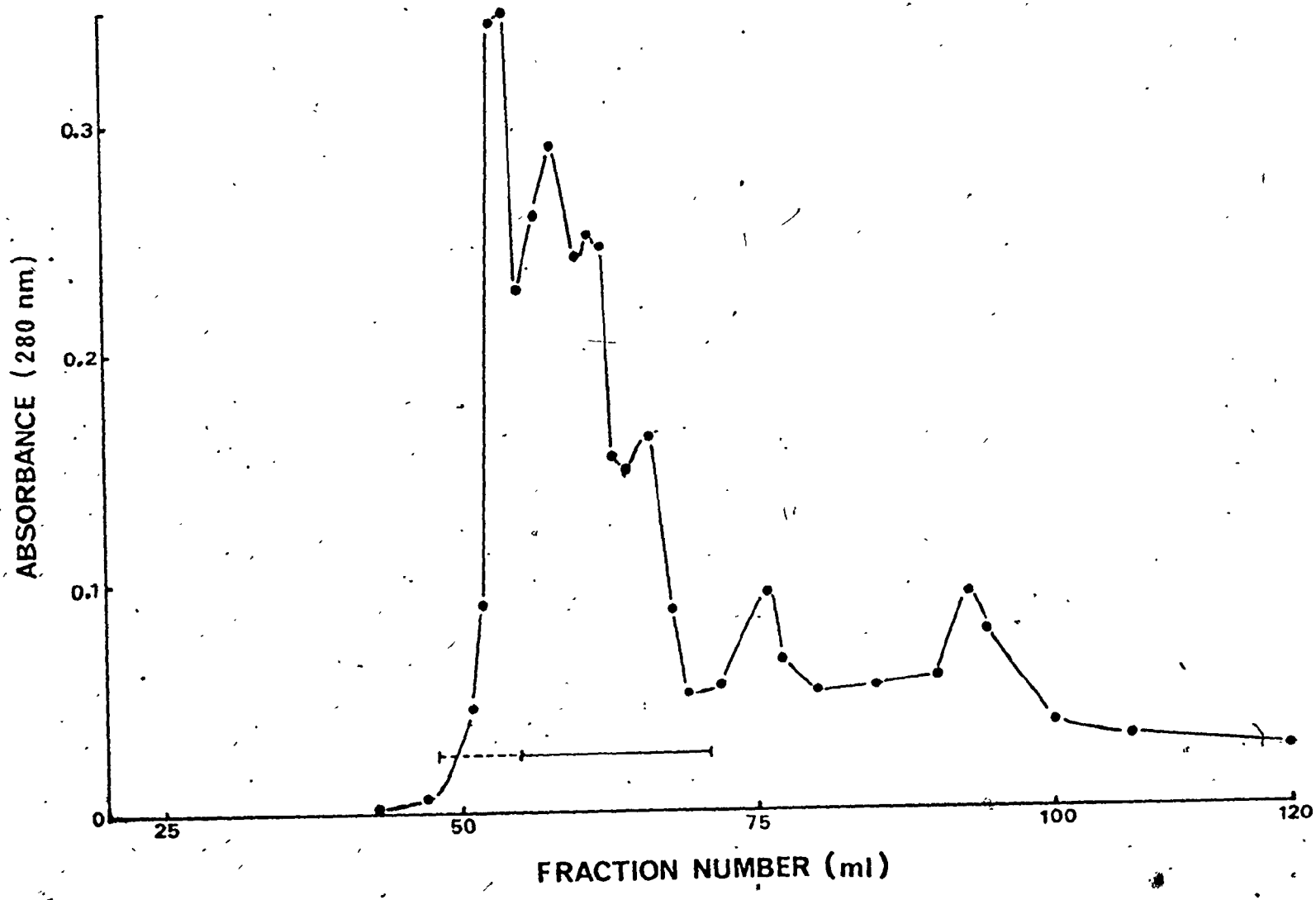

A
B
C
D
E

Figure 19. Separation of X1 and X2 on a Sephadex G-75 column.

An RBER-Txla extract was lyophilized and washed sequentially with butanol and acetone. The remaining precipitate was dissolved in water. One ml of this extract containing 4.7 mg of protein was eluted from a 1.5 x 82 cm Sephadex column with 0.5% SDS at an elution rate of 0.2 ml/min. The fractions obtained were analysed for antigens X1 and X2.

←------→ indicates X1

←———→ indicates X2


ABSORBANCE (280 nm)
0.3
0.2
0.1
0
25
50
75
100
120
FRACTION NUMBER (ml)

When an RBER-Tx2 extract, from which Triton X-100 had been removed, was eluted from a Sephadex G-100 column (Figure 20), separation of protein components was relatively good in contrast to the Sephadex G-75 elution of RBER-Tx1a. Antigen X2 was detected in a relatively large number of fractions (from 89 ml to 139 ml). Figure 21 is a photograph of the electrophoretic pattern obtained in SDS-polyacrylamide gels, with the fractions corresponding to volumes of 117 and 129 ml. Only three and four bands respectively were detected in the two fractions. For comparative purposes the electrophoretic profile of the extract applied to the column is also included.

Gel filtration of RBER-Tx2 extracts on Sephadex G-150 did not give as good separation as did the filtration on Sephadex G-100. The purest fraction containing X2 had 6 detectable bands after electrophoresis in SDS-polyacrylamide gels.

Triton X-100 was removed from RBER-Tx2 extracts by precipitation of the proteins with 9 volumes of acetone. The precipitates were solubilized in warm water. This procedure was much faster than lyophilization and washing with butanol and acetone. Also the antigen yield seemed to be about the same for both procedures. An RBER-Tx2 extract, from which the Triton X-100 had been removed by acetone precipitation, was eluted from a Sephadex G-100 column (equilibrated with 0.5% SDS). It will be seen from Figure 22 that X2 could be detected in a large volume (75 ml). The electro-

Figure 20. Elution pattern of an RBER-Tx2 extract (lyophilized and washed with butanol and acetone) on Sephadex G-100.
One ml of extract containing 3.4 mg of protein was eluted with 0.5% SDS from a 1.7 x 91 cm Sephadex G-100 column at a flow rate of 0.2 ml/min. The fractions obtained were analysed for X2.
——— indicates X2.

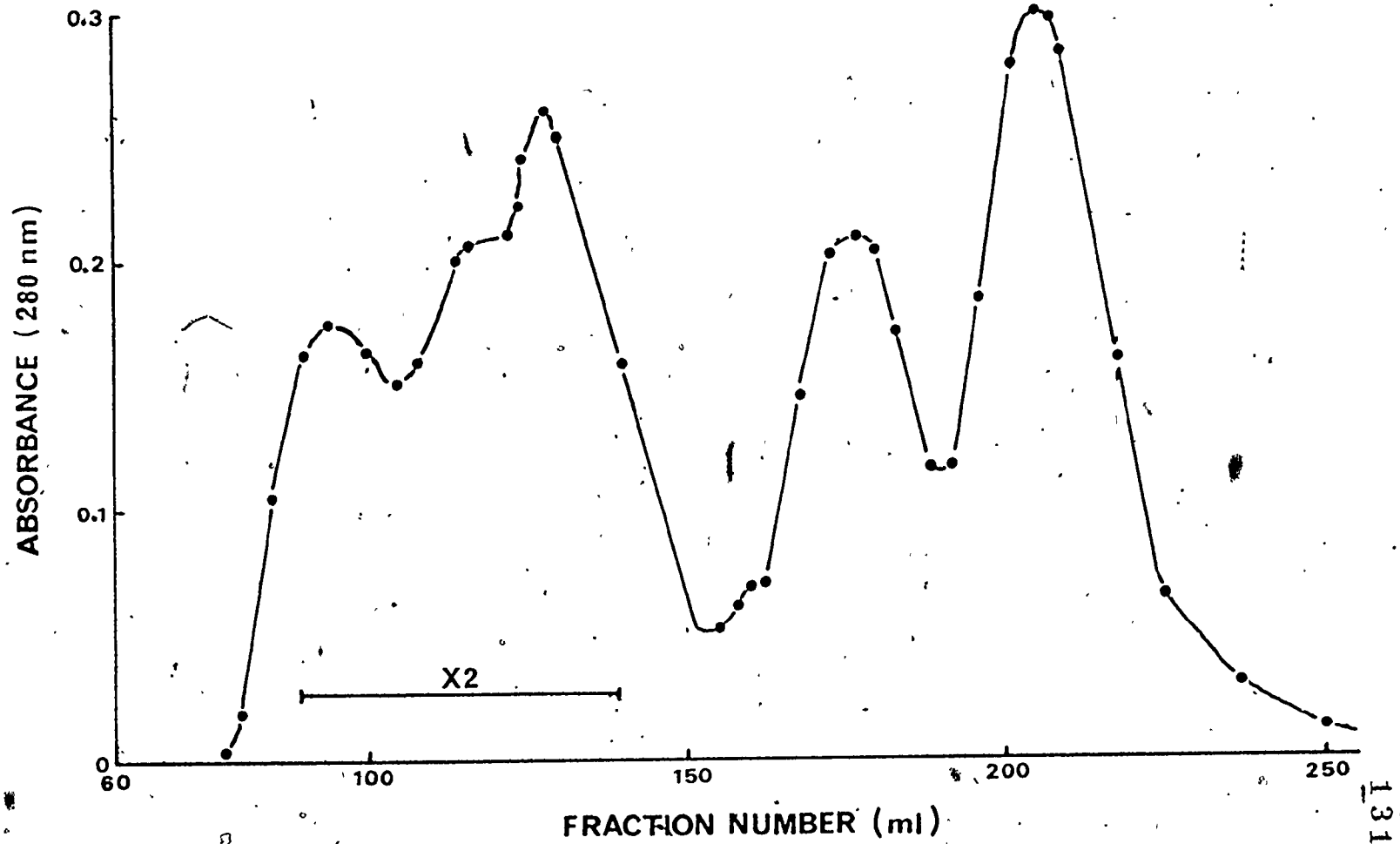

0.3
0.2
0.1
0
ABSORBANCE (280 nm)
X2
60
100
150
200
250
FRACTION NUMBER (ml)

Figure 21. Analytical SDS-polyacrylamide gel electrophoretograms of RBER-Tx2 before and after elution from Sephadex G-100.

The RBER-Tx2 was lyophilized, sequentially washed with butanol and acetone, and suspended in water. A and B represent the patterns obtained with fractions eluted at volumes of 117 and 129 ml respectively (see Figure 20). C represents the pattern obtained with the RBER-Tx2 extract before elution.

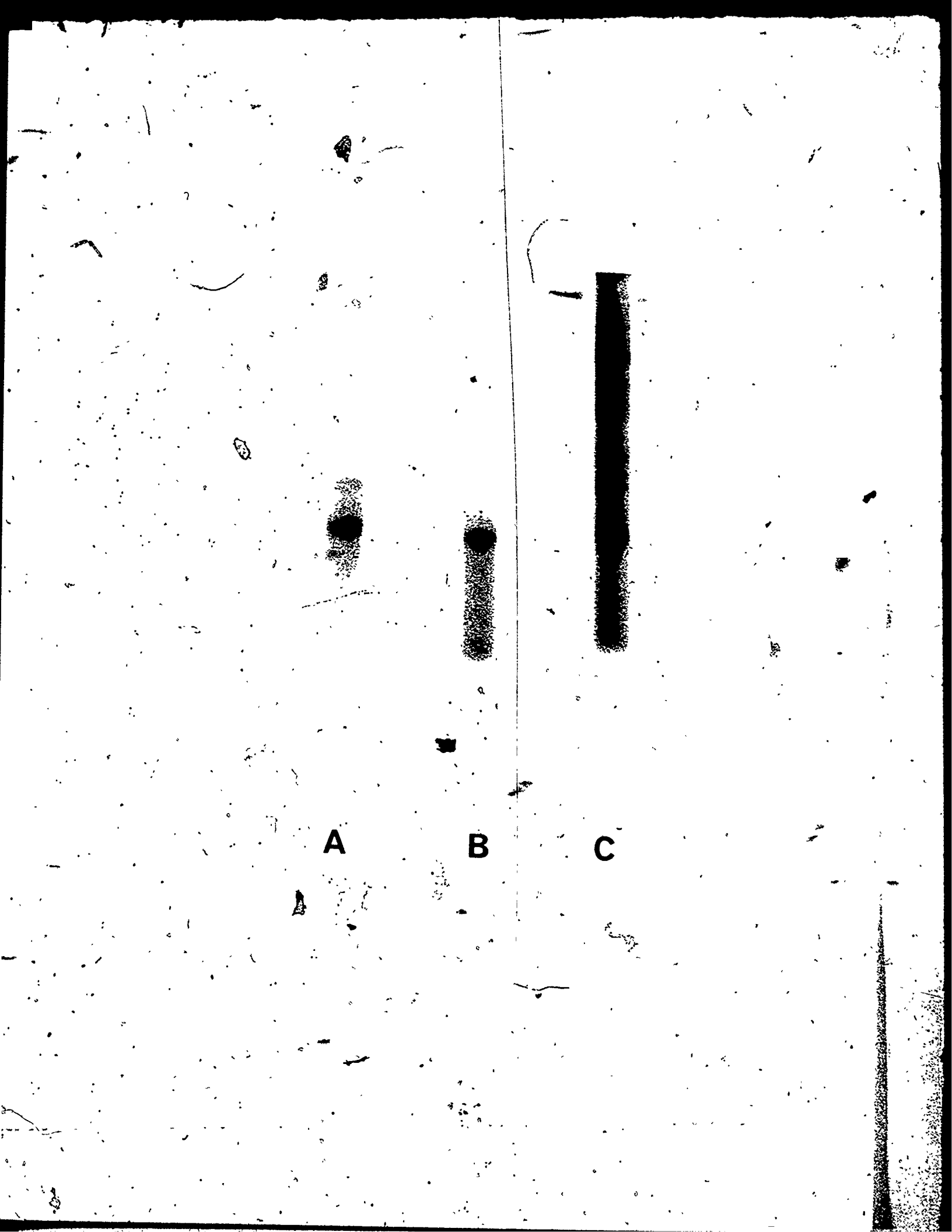
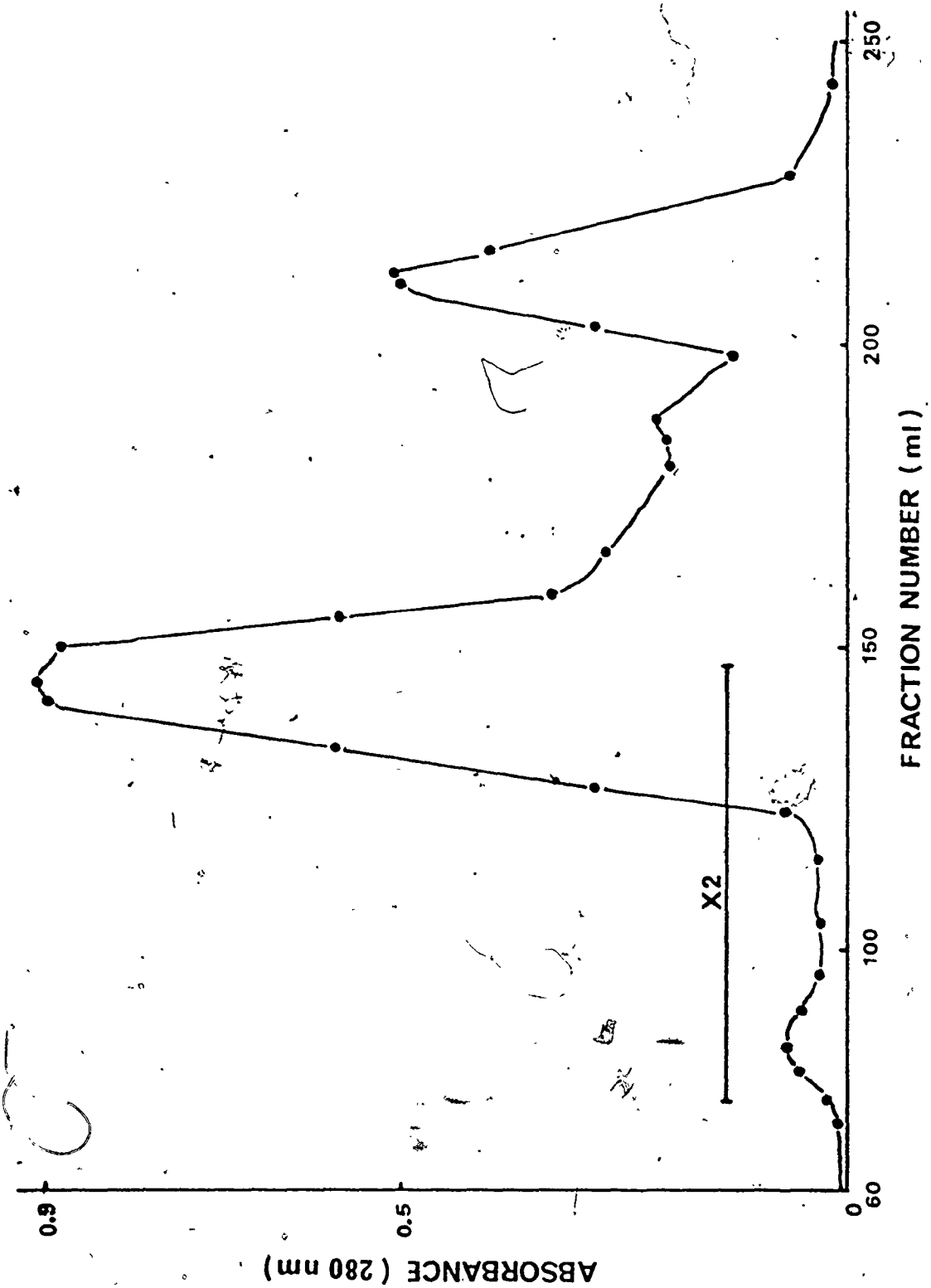

A
B
C

Figure 2. Elution pattern of an RBER-Tx2 extract (acetone precipitate) on Sephadex G-100. One ml of extract containing 3 mg of protein was eluted with 0.5% SDS from a 1.7 x 91 cm Sephadex G-100 column at a flow rate of 0.2 ml/min. The fractions obtained were analysed for X2. ⟷ indicates X2.


ABSORBANCE (280 nm)
0.9
0.5
0
60
100
150
200
250
FRACTION NUMBER (ml)
X2

phoretic patterns obtained on SDS-polyacrylamide gels (Figure 23) show that the fractions corresponding to volumes of 108 and 124 ml contained at least 4 and 5 polypeptides, respectively.

3.9 QUANTITATION OF ANTIGEN X2 BY SINGLE RADIAL IMMUNODIFFUSION

Quantitative single radial immunodiffusion was used in an attempt to quantitate the yield of antigen X2 in Triton X-100 extracts of washed RSM and RBER. The assay was performed as described in Materials and Methods, using anti-RBER serum B incorporated in the agarose. Since antigen X2 was not available in pure form, it was decided to use an RBER-Tx1a extract as standard and to assign an arbitrary value of one unit of X2 per µl of this extract. A standard calibration curve was obtained by measuring the diameters of the precipitin circles formed around the wells containing increasing volumes of standard RBER-Tx1a.

The concentration of X2 in Triton X-100 extracts of washed RSM and RBER was then determined. Table 4 is a summary of the protein and units of X2 obtained in the various extracts. It will be seen that slightly more protein was solubilized in the RSM-Tx1a and Tx2 extracts than was solubilized in the RBER-Tx1a and Tx2 extracts. The overall yield of antigen X2 was slightly higher in the Triton extracts of RBER, in comparison to the Triton extracts of RSM. This indicates that the specific activity (units X2 per mg protein)

Figure 23. Analytical SDS-polyacrylamide gel electrophoretograms of RBER-Tx2 (acetone precipitate) before and after elution from Sephadex G-100.

The RBER-Tx2 was precipitated with 9 vol of acetone and the precipitate was suspended in water. Electrophoresis was carried out, in 10% acrylamide gels containing 0.1% SDS, for 4-5 hrs at 8 mA/gel.

A and B represent the patterns obtained with fractions eluted at volumes of 108 and 124 ml respectively (see Figure 22).

C represents the pattern obtained with the RBER-Tx2 extract before elution.

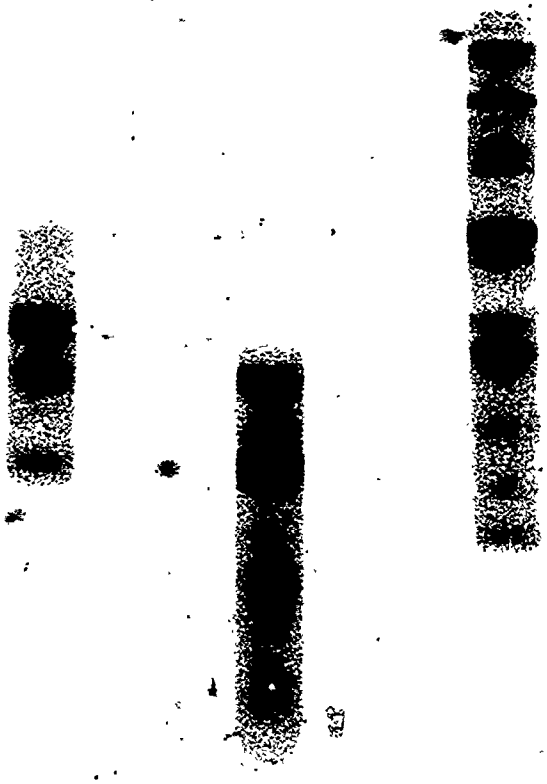

A
B
C

TABLE 4. Total protein and antigen X2 obtained from 20 mg of "washed" RSM and RBER protein by extraction with Triton X-100[a].

				ACETONE PRECIPITATES[b]			
				Water soluble		Water insoluble[c]	
	Extract	Total Protein (mg)	Total Units X2[d]	Total Protein (mg)	Total Units X2	Total Protein (mg)	Total Units X2
RSM							
	Tx1a	2.79	463	0.92 (33)[e]	79 (17)	0.71 (25)	676 (147)
	Tx1b	0.62	371	0.11 (18)	76 (20)	0.11 (18)	187 (50)
	Tx2	4.15	1015	0.21 (5)	452 (45)	0.19 (5)	278 (27)
	Total	7.56	1849	1.24 (16)	607 (33)	1.01 (13)	1141 (62)
RBER							
	Tx1a	2.04	679	1.30 (64)	333 (49)	0.11 (5)	336 (49)
	Tx1b	0.79	332	0.32 (41)	85 (26)	0.03 (4)	0 (0)
	Tx2	3.90	949	0.69 (18)	610 (64)	0.15 (4)	323 (34)
	Total	6.73	1960	2.31 (34)	1028 (52)	0.29 (4)	659 (34)

(a) The results represent an average from three extractions.
(b) Triton extracts precipitated with 9 vol of acetone.
(c) The water insoluble precipitates were solubilized in SDS.
(d) One µl of standard (RBER-Tx1a) was arbitrarily assigned one unit of X2.
(e) Figures in parentheses represent percentage recovery.

is slightly higher in the RBER extracts.

The pellets remaining after the sequential Triton X-100 extracts were suspended in 1% SDS. By immunodiffusion analyses, antigen X2 can still be detected in these SDS extracts of RSM and RBER. Quantitation of antigen X2 in these extracts was not attempted since the pellets could not be completely solubilized, even after incubation in a boiling water bath for 10 min.

The Triton X-100 extracts were precipitated with 9 volumes of acetone. The precipitates that formed were suspended in 0.5 ml of water and incubated at 37°C for 30 min. After centrifugation, the water insoluble pellets were solubilized by suspension in 1% SDS and incubation at 37°C for 30 min.

Table 4 summarizes the yields of protein and antigen X2 obtained with the water soluble and water insoluble acetone precipitates. It will be seen that a larger percentage of the initial protein could be recovered in the individual water soluble fractions of RBER as compared to the water soluble extracts of RSM. About 34% of the total RBER Triton extract protein was recovered in the water soluble fraction in contrast to only 16% for the RSM extracts.

Only 33% of total antigen X2 was recovered in the water soluble fraction of RSM extracts, while 52% of the antigen could be recovered in the water soluble fraction of RBER. Most of this difference can be accounted for by the low recovery (17%) of X2 in the RSM-Txla water soluble acetone precipitates.

A total of 13% of the initial RSM protein and 4% of the initial RBER protein were recovered in the water insoluble fractions of acetone precipitates. Most of this difference can be accounted for by the relatively high recovery (25%) obtained for the RSM Txla extract.

Sixty-two percent of the total antigen X2 could be recovered in the RSM water insoluble fraction of acetone precipitates in contrast to 34% recovery in the RBER water insoluble fractions. The high recoveries of X2 in the RSM-Txla (147%) and RSM Txlb (50%) are difficult to explain. It is suspected that the initial yield (463 units) of X2 in RSM-Txla extracts is probably low. These extracts are somewhat cloudy when kept cold (4°C); however, the extracts become clear when warmed gently. From gel filtration experiments, it was found that X2 is present as large aggregates in Txla extracts. It is possible that not all antigenic determinents are exposed in these aggregates and that not all of the X2 activity can be detected. However, after solubilization in SDS, which is known to dissociate the aggregates, most of the X2 activity may have been detected.

The total protein recoveries in the acetone precipitates (water soluble and insoluble) were 29% and 38% for the RSM and RBER respectively. The remainder of the protein can be detected in the acetone supernatant. In contrast, 95% and 86% of antigen X2 could be recovered in the acetone precipitates of RSM and RBER respectively. Therefore, this indicates that antigen X2 has been purified by acetone precipitation.

The specific activities for the Tx2 extracts in units of X2 per mg protein are: RBER whole extract : 243;

RBER water soluble precipitate : 884;

RBER water insoluble precipitate : 2153;

RSM whole extract : 245;

RSM water soluble precipitate : 2152;

RSM water insoluble precipitate : 1463.

Similarly it can be calculated that the RSM-Tx1a and Tx1b, and RBER-Tx1a water insoluble acetone precipitates have high X2 specific activities.

Extracts obtained by acetone precipitation of Triton extracts were analysed by PAGE on 10% polyacrylamide gels containing 0.1% SDS. The extracts were suspended in 1% SDS and incubated in a boiling water bath for 3 min. It will be seen from Figure 24A that the water soluble fractions, obtained from acetone precipitates of RBER-Tx1a, Tx1b and Tx2 extracts, contain a large number of components that can be detected in PAGE patterns. There are only about half as many detectable components in these extracts as compared to the PAGE patterns obtained from SDS extracts of RBER (see Figure 10). Also a band corresponding to a molecular weight of 28-32,000 daltons, tentatively labelled as antigen X2 is a major component of the water soluble fraction obtained from acetone precipitates of Triton extracts.

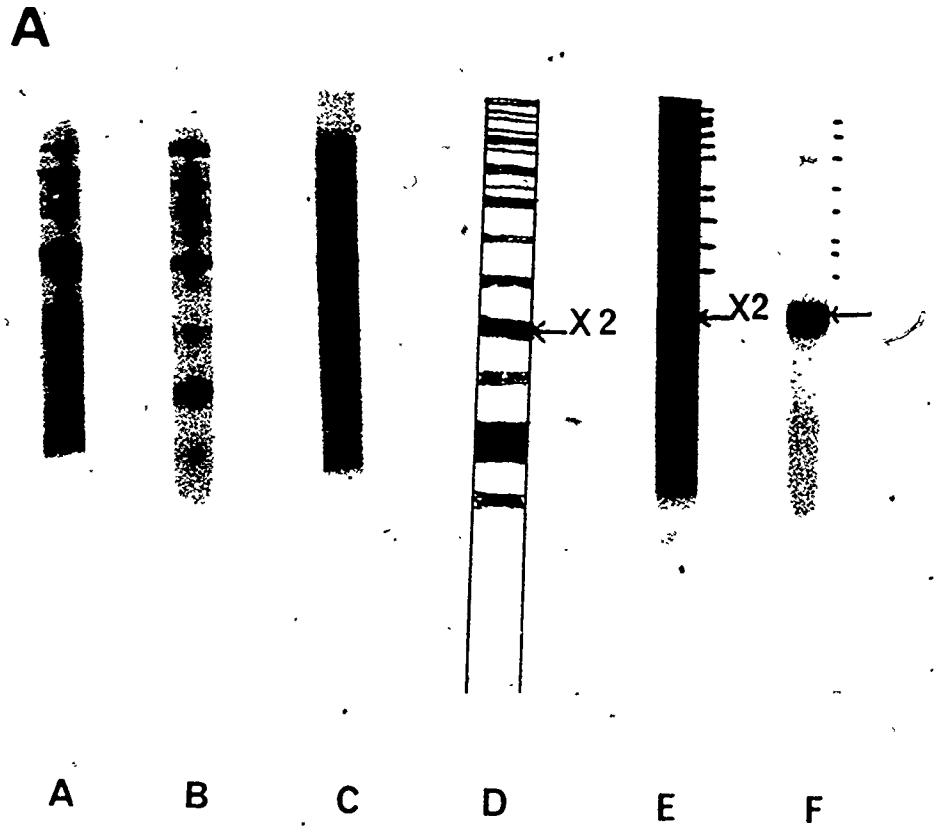
For RSM, the pattern is different in that the band corresponding to antigen X2 seems to be the predominant component of the water soluble acetone precipitates. A smaller amount of protein was used for the RSM-Tx2 gel, to

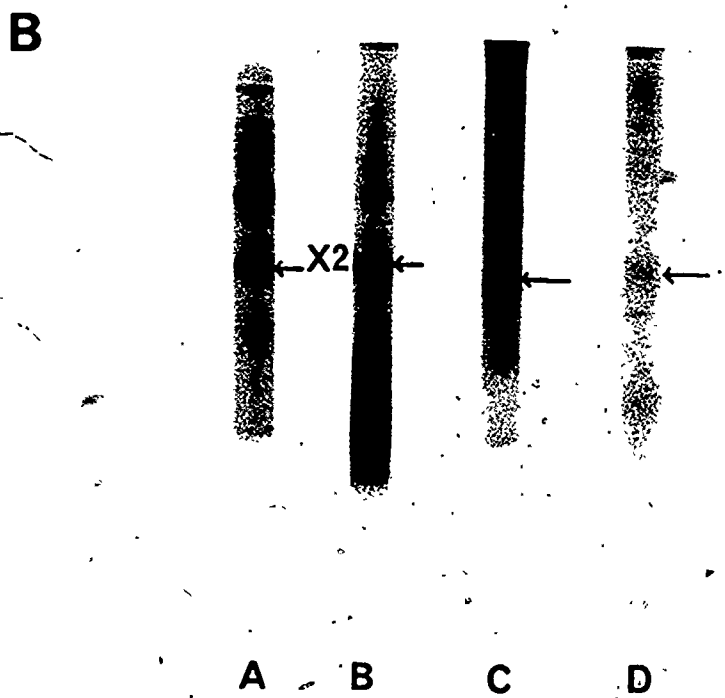
Figure 24. SDS-polyacrylamide gel electrophoretograms of acetone precipitates obtained from RSM and RBER Triton X-100 extracts.

Electrophoresis was carried out in 10% acrylamide gels (0.1% SDS) for 4-5 hr at 8 mA/gel. The bands labeled X2 correspond to a molecular weight of 28-32,000 daltons.

A. Patterns obtained with the water soluble fraction of acetone precipitates from Triton X-100 extracts. The samples in the gels were :
(A) RBER-Tx1a, 100 µg; (B) RBER-Tx1b, 40 µg; (C) RBER-Tx2, 74 µg; (D) RSM-Tx1a, 74 µg; (E) RSM-Tx1b, 37 µg; (F) RSM-Tx2, 42 µg. The small bars indicate weakly stained bands.

B. Patterns obtained with the water insoluble fraction of acetone precipitates from Triton X-100 extracts. These samples were solubilized in 1% SDS. The samples in the gels were:
(A) RBER-Tx1A, 48 µg; (B) RBER-Tx2, 45 µg; (C) RSM-Tx1A, 74 µg; (D) RSM-Tx2, 46 µg.


A
X 2
X2
A
B
C
D
E
F


B
X2
A
B
C
D

stress the point that the band due to X2 is the major component. Bars indicate the location of lightly stained bands. Due to the heavy background staining in the RSM-Tx1b gel, bars were used to indicate the location of bands that are not clearly seen in the photograph. The pattern for RSM-Tx1a was drawn since background staining was too heavy to obtain a good photograph. The heavy background staining in the RSM-Tx1a and Tx1b was obtained when extracts prepared from three separate RSM preparations were used.

Figure 24B shows the PAGE patterns obtained with the water insoluble fractions obtained from acetone precipitates of Triton extracts. Arrows indicate the location of lightly stained bands. It will be seen that there are a number of detectable components but, the band corresponding to antigen X2 is a major component of the RBER-Tx2 and of the RSM-Tx1a and Tx2 extracts.

The position and intensity, of the band corresponding to X2, are not changed if 2-mercaptoethanol is added to the extracts.

It should be mentioned that the protein yield was very low for the RSM-Tx1b water soluble and water insoluble acetone precipitates, for the RBER-Tx1a water insoluble acetone precipitates, and for the RBER-Tx1b water insoluble acetone precipitates. This did not present a problem for the quantitation of X2 but the protein determinations for these extracts were subject to a large error.

CHAPTER 4. DISCUSSION

4:1 PURITY OF SUBSYNAPTOSOMAL FRACTION F2 (RSM)

A number of problems are encountered when synaptic membranes are isolated. Firstly, due to the cellular heterogeneity of the brain, the SM fraction may be contaminated with glial, axonal, dendritic, and perikaryal plasma membranes. Secondly, there may be extensive contamination with other subcellular membranes such as microsomal, mitochondrial, and lysosomal.

A number of reports have indicated that the purity of SM preparations depends primarily on the purity of the synaptosome fraction (Morgan *et al.*, 1971; Gurd *et al.*, 1974). This stems from the observation that the sedimentation density, in sucrose, of many contaminating membranes (microsomal, glial, axonal and outer mitochondrial membranes) overlap the density of SM. Therefore, these contaminating membranes should be eliminated as much as possible before synaptosomes are lysed and placed on sucrose gradients. We therefore decided to isolate rat brain synaptic membranes (RSM) according to the method of Cotman and Mathews (1971). This procedure makes use of Ficoll gradients to isolate synaptosomes, which are reported to be less contaminated with glia and mitochondria than synaptosomes isolated on sucrose gradients (Autilio *et al.*, 1968; Flexner *et al.*,

1971; Appel *et al.*, 1972; Cotman, 1972). To decrease the microsomal contamination, the crude mitochondrial fraction was washed once according to the method of Gurd *et al.* (1974) and Morgan *et al.* (1971).

According to the relative specific activity of the subsynaptosomal fraction F_2, for the mitochondrial marker SDH, this fraction could contain approximately 3% of mitochondrial membranes (Table 1).

Similarly, microsomal contamination of the RSM can be calculated to be in the order of 38% using the results obtained with NADPH: cytochrome c reductase. These results correlate quite closely to those of Gurd *et al.* (1974). However, Gurd *et al.*, (1974) found that NADPH:cytochrome c reductase was not uniformly distributed among microsomal membranes, but was concentrated 2 to 3 fold in the least dense fraction of microsomal membranes, namely the fraction that would band with synaptic membranes during density gradient centrifugation. If one assumes that the activity of the least dense microsomal fraction is 2 to 3 times the activity of the total microsomal fraction, then the RSM used for the present work would have contained between 13 and 19% of microsomal membranes.

The assay for cholinephosphotransferase, a microsomal enzyme necessary for the synthesis of choline phospholipids, indicates that the RSM contained approximately 10% of microsomal membranes. This enzyme would seem to be a more useful indicator of microsomal contamination of the subcellular

fractions since the activity is clearly segregated in the microsomal fraction. Since cholinephosphotransferase was enriched approximately 4.2 fold in the microsomal fraction, this enzyme may be more representative of the microsomal fraction than NADPH:cytochrome c reductase which was enriched only 2 fold.

A number of reports indicate that the *de novo* synthesis of nitrogen containing phospholipids, in liver, takes place only in smooth and rough endoplasmic reticulum (Wilgram and Kennedy, 1963; McMurray and Dawson, 1969; Sarzala *et al.*, 1970; Van Golde *et al.*, 1971, 1974; Dennis and Kennedy, 1972). This would also seem to be the case in brain (Porcellati *et al.*, 1970; Miller and Dawson, 1972a, b; Jungalwala, 1974; Toews *et al.*, 1975). Since the *de novo* production of phosphatidyl choline must proceed via the cholinephosphotransferase step (Ansell and Spanner, 1967), the choice of this enzyme as an appropriate marker for endoplasmic reticulum seems reasonable and the results obtained support the above evidence. The present results also support the observations of Miller and Dawson (1972a) which indicated that synaptosomes are not able to incorporate ^{32}Pi or ^{14}-[Me]-choline into lipids.

Since axons and nerve endings contain endogenous smooth endoplasmic reticulum (Droz, 1975; Droz *et al.*, 1975; Tsukita and Oshikawa, 1976), it is unlikely that SM can be prepared free of microsomes by density gradient centrifugation.

The RSM used for the present work were also analysed for the presence of myelin, by immunodiffusion analyses based

on the use of a rabbit anti-rat-myelin basic protein antiserum. Purified rat myelin basic protein and Triton X-100 extracts of purified myelin of known protein concentration were used as standards. This method can detect less than 1% myelin in extracts containing 1 mg of membrane protein per ml (MacPherson *et al.*, unpublished results). Using this method, myelin could not be detected in Triton extracts of RSM.

Electron microscopic analysis of the RSM used for the present study revealed the presence of only vesicles and sheets of smooth membrane (Figure 2). The appearance of this fraction was comparable to the electron micrographs presented by Cotman and Mathews (1971), Morgan *et al.*, (1971) and Gurd *et al.* (1974). It should be noted that there is difficulty in identifying the source of membranes seen in electron micrographs of isolated SM (Jones, 1975).

Since enzyme markers for glial and perikaryal plasma membranes, outer mitochondrial membranes, and axonal membranes were not examined, it is difficult to make any conclusions as to the contamination of the RSM fraction with these membranes. In this regard, Lemkey-Johnston and Dekirmenjian (1970) have concluded that significant amounts of axonal membranes could be present in SM preparations. Also, Morgan *et al.*, (1972) provided evidence that their SM preparation could have contained 5-10% of glial membranes.

It should be noted that the specific activity of the fraction most enriched with an organelle (ie. mitochondria) is used to calculate contamination of any other fraction

with this organelle. Since the enriched fraction (i.e. mitochondrial fraction) is itself not completely pure, it is obvious that any calculations of contamination are maximal values and that contamination is in effect probably lower than indicated. This is especially true for calculating microsomal contamination since the microsomal fraction is very heterogeneous.

4.2 SYNAPTOSOME SWELLING

Keen and White (1970, 1971) showed that synaptosomes swell and shrink in media of different tonicity and have selective permeabilities for various ions. The degree of synaptosome swelling can be followed spectrophotometrically by a light scattering technique. Raiteri *et al.* (1972, 1973, 1974) using the Keen and White method, reported that in the presence of complement, anti-synaptosomal antibodies inhibited synaptosomal swelling in 0.4M glycerol. Moreover, they found that the degree of inhibition of synaptosomal swelling may be reliably determined and was a quantitative measurement of the concentration of antibody directed against one or more antigens of the external synaptosomal membrane.

In order to learn where synaptic membrane antigens are located in the membrane, it is highly desirable to use a method for measuring the change in a property of the intact synaptosomal membrane that can be caused by anti-synaptic membrane antibodies. Thus it was decided to investigate the reliability of this relatively simple method devised by

Raiteri *et al.* (1972) to measure the ISS antibody in the anti-synaptic membrane sera raised in this laboratory by hyperimmunizing rabbits with purified RSM.

The initial experiments confirmed the observation that a decrease in $O.D._{520}$ occurs as synaptosomes, previously incubated with normal rabbit serum, swell when suspended in 0.4M glycerol. Swelling in the presence of normal serum is expressed as 100% swelling, and provides a standard which can be used to calculate the ISS activity of antisera. The raw data obtained from optical density measurements, when antisera are used, must be corrected since it was observed that swelling of synaptosomes decreases in linear fashion when synaptosomes are incubated with increasing volumes of normal serum. This effect does not appear to have been reported previously. It could be due to a number of factors such as the presence of low levels of anti-synaptosome antibodies in normal serum or the presence of nonspecific factors which inhibit synaptosome swelling or cause lysis of synaptosomes.

The present report (using anti-RSM serum) confirms the results of Raiteri *et al.* (1972) who demonstrated that anti-synaptosome serum inhibits synaptosome swelling and that this inhibition is complement dependent. An ISS of about 75% could be detected (Figure 3) and this inhibition could not be increased by using larger amounts of antiserum. Raiteri *et al.* (1973) obtained a maximum inhibition of approximately 80-85%. Howe *et al.* (1977) found that anti-SM serum inhibited 80-85% of synaptosome swelling.

Raiteri *et al.* (1972, 1973) presumed that the observed ISS activity was due to synaptosomal structural damage caused by antibodies in the presence of complement but this has not been proven. However, De Robertis *et al.* (1966, 1968) showed by electron microscopy that anti-synaptosome sera lysed nerve-endings *in vitro* in the presence of complement.

The results of Howe *et al.* (1977) indicate that 100 and 200 µl of their anti-SM serum per mg of synaptosomal protein inhibited 60 and 85% respectively of the total synaptosome swelling. The present results indicate that 100 µl of anti-RSM serum per mg of synaptosomal protein inhibit 70% of the swelling, which is the maximum inhibition that can be obtained. Therefore, on a direct comparison the antiserum used for the present study would seem to be equivalent to the one used by Howe *et al.* Howe *et al.* (1977) corrected their swelling values by using the $O.D._{520}$ decrease, obtained with synaptosomes suspended in 0.32M sucrose (no glycerol), as zero swelling. Their 100% ISS therefore, probably corresponds to the 70% ISS of the present study. The results obtained by Howe *et al.* (1977) indicate that in excess of 250 µl of antiserum per mg of synaptosomes would be required to obtain 100% ISS. If this interpretation is valid, the present antiserum could be 2-3 times more active than the antiserum used by Howe *et al.*

Figure 7 is a summary of the results obtained after anti-RSM serum had been absorbed with various amounts of RSM, RBM or synaptosomal proteins. It can be seen that 8 mg of RSM protein completely absorbed the ISS antibody activity.

Synaptosomal protein does not seem to be as effective an absorbent as RSM protein. This is not surprising since synaptoplasm accounts for a large proportion of the total synaptosomal protein and would not be expected to have much effect on antibodies directed against antigens located on the synaptosome surface. Raiteri *et al.* (1974) indicate that absorption of anti-synaptosome serum with synaptosomes completely decreases ISS activity but no mention is made as to the amount of protein used as absorbent.

The absorption pattern obtained with increasing amounts of RBM, is interesting because, even after the use of 20 mg of RBM protein per ml of serum, 5-10% of the ISS antibody activity was still detected. Possibly, this residual activity is directed against antigens specific for the synaptosomal surface. Even if we disregard this residual antibody activity, it can be seen that RBM are less effective than RSM for absorption of antibodies responsible for ISS. Raiteri *et al.* (1974) obtained similar results. When an anti-synaptosome serum was absorbed with 2 mg of myelin, 2.4 mg of mitochondria, or a mixture of the two absorbents (per ml of serum), 69, 50, and 44% respectively, of the ISS antibody activity could still be detected. Synaptosomes rapidly absorbed all the ISS activity (Raiteri *et al.*, 1974).

Results from complement fixation assays (Table 2) tend to parallel the ISS results. A maximum titre of 320 was obtained with unabsorbed anti-RSM serum when analysed with either RSM, RBM or synaptosomes. In support of this finding

is a report by Lim and Hsu (1971) which demonstrated that on a protein basis, microsomes and synaptic membrane fixed identical amounts of complement when tested with an anti-RSM serum.

After absorption with various amounts of RSM or RBM the complement fixation titre of the anti-RSM serum quickly drops to insignificant levels. The major difference between these results and the ISS results seems to be that absorption with 6 mg of RBM decreased complement fixation titres to almost zero while ISS activity has only been decreased by 60%. This is partially in accord with the results of Raiteri *et al.* (1974) which indicate that, after absorption of an anti-synaptosome serum with various quantities of myelin or mitochondria, the percentage decrease in complement fixation titres is always greater (almost twice) than the percentage decrease in ISS activity. This suggests that the swelling assay has greater sensitivity and specificity in comparison to the complement fixation assay. In this regard, Raiteri *et al.* (1973) suggested that the synaptosome swelling assays and the complement fixation test are not alternatives since no correlation could be demonstrated between them. In an analysis of 12 different sera, they found that sera with high complement fixation titres often had low ISS titres and vice-versa.

Figure 8 indicates that immunodiffusion analyses are not as sensitive as ISS tests since absorption with low amounts of RSM, RBM and synaptosomes very quickly reduced the

antibody to undetectable levels. A good example is pattern L where, after absorption of anti-RSM serum with 8 mg of synaptosomal protein, no precipitin lines were formed while 55% of ISS activity could be detected.

Figure 8 also demonstrates that synaptosomes are more effective than RBM or RSM in absorbing anti-soluble components from the serum. This is not surprising since synaptosomes contain synaptoplasm.

Thus, the ISS results can be explained in a number of ways as follows: (1) the RSM fraction used as immunogen was heavily contaminated with microsomal membranes, (2) microsomes present in the synaptosome fraction may swell, (3) there may be extensive contamination of the RBM fraction with synaptosomes or RSM, or (4) there may be extensive sharing of antigenic determinants between the RSM and RBM.

The first two suggestions have been addressed by Raiteri *et al.* (1974). They had used the synaptosomal fraction as immunogen. Since this fraction is known to be contaminated with free mitochondria and since synaptosomes contain their own (intraterminal) mitochondria, one would expect specific anti-mitochondria antibodies in the serum. Raiteri *et al.* suggest that the only antibodies being measured by this test are those directed against antigenic determinants located on the surface of synaptosomal membranes. Any specific anti-mitochondria antibodies present in the serum should not affect the swelling properties of synaptosomes.

Such an explanation would be equally relevant to microsomal contamination of the RSM fraction used as immunogen in

the present study. The anti-RSM serum probably contains a minimal level of antibodies directed specifically against RBM since enzyme assays indicate that the RSM used to immunize rabbits contained approximately 10% of microsomal membranes.

In his interpretation, Raiteri has assumed that only synaptosomes swell in 0.4M glycerol. Raiteri *et al.* (1972) stated that myelin and mitochondrial fractions swell very little in glycerol but did not report any data. In contrast (Table 3) indicates that there is substantial swelling of the microsomal and mitochondrial fractions in 0.4M glycerol and that anti-RSM serum inhibits this swelling. The problem is a very significant complicating factor in interpreting the results since the microsomal fraction seems to swell about 160% as much as the synaptosome fraction.

The third suggestion presented to explain the absorption results is that there may be extensive contamination of the microsomal fraction with synaptic membrane or synaptosomes. In the majority of cases where plasma membrane markers were measured in brain subcellular fractions, the results indicate heavy contamination of microsomal fractions with plasma membrane. It is to be expected that some of the plasma membrane present in RBM would be synaptic membrane. In this respect, Kataoka and De Robertis (1967) have demonstrated that a large number of small synaptosomes sediment with the microsomal fraction. In the present report, this contamination was minimized by resuspending the microsomal fraction in sucrose and discarding the pellet obtained from recentrifuging

at 17,000g. One would expect any synaptosomes or RSM present in the RBM fraction to slowly absorb all specific anti-synaptosome antibodies. Therefore strong emphasis is not placed on the residual antibody activity that can be detected after absorption of anti-RSM serum with RBM.

The fourth suggestion is that there may be extensive sharing of antigenic determinants present on RSM and microsomes. The discussion to be presented in a later section, concerning the presence of antigens X1 and X2 on RSM and RBM is relevant and will not be presented here except to mention that there is extensive evidence supporting the suggestion that RSM and RBM share antigenic components. The results presented in Table 2 and Figure 8, support sharing of antigenic components but do not exclude the possibility that RSM contamination of the microsomal fraction could contribute to the results.

Thus, it is suggested that a large proportion of the antibodies responsible for ISS are directed against antigenic determinants shared by RSM and microsomes. Some of the ISS activity may be explained in terms of cross contamination of subcellular fractions and possibly in terms of microsomal swelling. Finally, a small portion of ISS antibodies may be directed against specific RSM components.

The ISS activity of an anti-RBM serum (Figure 9) is difficult to interpret. It is not surprising that an anti-RBM serum can inhibit synaptosome swelling since antibodies directed against components shared by RSM and microsomes

would be expected to affect synaptosomes. Also, antibodies would have been engendered by the synaptosomes which contaminated the microsomal fraction. Thus, absorption of antiserum with RBM would be expected to decrease ISS antibody activity leaving a small degree of activity due to antibodies directed specifically against synaptosomes. It was surprising that 30% of ISS antibody remained after absorption with RBM. This can not be explained in terms of microsomal swelling since the absorption with RBM would remove any antibodies directed against microsomes. The results therefore seem to indicate that the antibodies remaining, after absorption with RBM, are probably directed against specific synaptosomal components. This is surprising since one would expect that synaptosomes present in the microsomal fraction would slowly remove any specific anti-synaptosome antibodies.

When RSM protein is used as absorbent, approximately 60% of ISS activity remained. One might speculate that some of this remaining activity could be due to the effect of specific anti-microsome antibodies on inhibition of microsome swelling. Since only 30% of total antibody activity can be detected after absorption of this same anti-RBM serum with RBM, it is possible that the difference in antibody activity (60% minus 30%) could be attributed to specific anti-microsome antibodies. That microsomes present in the synaptosome fraction could account for 30% of the observed swelling is not out of line with enzyme studies which indicate that synaptosomes could be contaminated with approximately 20% of microsomal membranes.

Results obtained after absorption of anti-RBM serum with RSM could also be explained differently. The procedure for RSM preparation involves lysis of synaptosomes in 6 mM Tris-HCl, pH 8.1. It is possible that this treatment could remove components which are loosely bound to the synaptosome surface membrane. The anti-RBM serum would be expected to contain antibodies to these peripheral synaptosome components and absorption with RSM would not remove these antibodies.

A few final comments concerning the synaptosome swelling assay are in order at this point. It would seem that some doubt has been raised as to the validity of interpreting results only in terms of synaptosome swelling. It was shown that components present in the mitochondrial and microsomal fractions are able to swell when suspended in 0.4M glycerol. Swelling of the microsomal fraction could be due to endoplasmic reticulum and/or to contaminating membranes such as synaptosomes, neuronal plasma membrane, glial membranes or Golgi apparatus. It is possible that subfractionation of the microsomal fraction could give some indication as to the nature of the vesicular components actually responsible for the observed swelling.

Overall, the swelling assay would seem to be useful for the detection of specific and non-specific anti-synaptosome antibodies but interpretation of results should be done with caution. Interpretation of results is probably more complex than Raiteri *et al.* (1974) have indicated.

4.3 ANTIGENS X1 AND X2

The present results have confirmed the report of MacPherson *et al.* (1973) which demonstrates the importance of subjecting the membranes to sequential salt washes and of absorbing the antisera with the soluble fraction of brain. It is conceivable that the washing treatment could remove many proteins concerned with highly specific functions and which may be important antigenic components of RSM. For the present experiments it was deemed more important to remove non-specifically absorbed soluble fraction components than to preserve any theoretically specific peripheral membrane components.

Before discussing the immunochemical results, a few comments concerning the effect of Triton X-100 on immunoprecipitations are in order. According to reports by Crumpton and Parkhouse (1972) and by Bjerrum and Lundahl (1973), 2% Triton X-100 does not have any effect on immunoprecipitation although Qualtiere, Anderson and Myers (1977) reported that Triton X-100 does slightly inhibit the precipitin reaction. In the present experiments, it was found that concentrations of Triton X-100 above 20% change the intensity of precipitin lines but not their rate of appearance.

In the early stages of this project, RSM- and RBER-TxO extracts were used for immunodiffusion analyses. Two antigens, X1 and X2, could be detected in these extracts and both antigens seemed to be shared by the RSM and RBER. These antigens were considered to be integral components

of the synaptic membrane matrix and the microsomal membrane matrix because they were not removed from RSM or RBER by sequential washing with sodium salts.

At a later stage, the Triton extraction procedure was modified so as to obtain TxA and TxB extracts. The yield of antigens X1 and X2 was greater than had been obtained in the TxO extracts. It is suspected that some of the antigens were nonspecifically bound to the collodion membranes during the TxO preparation. Concentration by flash evaporation solved this problem. The modified extraction procedure indicated that X1 and X2 could be sequentially extracted from RSM and RBER. Therefore, the extraction procedure was modified further so as to obtain Tx2 extracts which contained X2 and not X1.

Treatment of RSM and RBER with butanol-H_2O yielded water phases in which antigen X2 could be detected. The butanol extract of RBER also contained antigen X1. About 18% of the total membrane protein was solubilized by this procedure. The antigenic activity, as measured by immunodiffusion analyses, decreased substantially when the extracts were stored at 4°C. This problem was solved by concentrating the water phases by flash evaporation in the presence of Triton X-100. The antigenic activities could then be preserved for several months.

Initially, immunodiffusion analyses with the Triton X-100 and butanol extracts were not very satisfactory since precipitin bands due to X1 and X2 developed at different

rates (see Figure 5). The incorporation of 5% aprotinin in the agarose appears to facilitate the diffusion of X1 and allows precipitin bands due to X1 and X2 to develop at the same rate. Aprotinin, a peptide protease inhibitor, which is used in immunodiffusion analyses of "sticky" proteins such as myelin basic protein or human SCP (MacPherson, personal communication) does not interfere with antigen-antibody reactions.

Antigens X1 and X2 derived from RSM were clearly found to be identical to the two most prominent antigens of RBER by immunodiffusion analyses. Moreover, preliminary experiments indicated that when anti-RSM IgG had been absorbed with enough RBER to remove all precipitating antibodies against RBER, the absorbed anti-RSM IgG solution no longer formed precipitin lines with extracts of RSM. These results demonstrate that X1 and X2 are components of both the synaptic membrane fraction and the microsomal fraction. The results also demonstrate that X1 and X2 are the major immunogens (in rabbits) of RSM and RBM.

Although the RSM used for immunization could have contained as much as 10% of RBM, it is improbable that the RSM and RBER could be immunologically identical as demonstrated at the level of immunodiffusion analyses unless the antigens X1 and X2 had originated in both membranes. This is supported by the quantitative studies which indicate that X2 can be obtained in equal quantity by Triton X-100 extraction of RSM and RBER.

To explain the quantitative results in terms of cross contamination, it can be assumed that the membrane which contains X2 should be present in approximately the same concentration in RSM and RBER. Since the RSM used for extraction of X2 could have contained 10% of RBM it is improbable that X2 would be a component only of endoplasmic reticulum. Estimations of synaptic membrane contamination in the microsomal fraction could not be made. In this regard, Kataoka and De Robertis (1967) reported that microsomal fractions contain a significant amount of small synaptosomes, but no quantitative data was presented. Synaptosomal contamination of the microsomal fraction, used for the present study, was minimized by re-centrifuging the RBM at 17,500 xg and discarding the pellet. It is improbable that the microsomal fraction could contain proportionately as much synaptic membrane as does the subsynaptosomal fraction F_2 and thus improbable that X2 could be a component only of the synaptic membrane; however, this possibility can not be excluded.

As an alternative, it can be proposed that X2 could be a component of a minor membrane fraction that equally contaminates both the RBM and RSM. This possibility can not be excluded but is unlikely since X2 was found to be the major immunogen of RSM and RBM. In this regard, preliminary immunohistochemical experiments, with anti-RBER serum B (using fluorescein-labelled goat anti-rabbit globulin), indicate that staining of myelin, astrocytes, and axons could not be

detected. Thus, these membranes, which may be minor contaminants of RSM and RBM, probably are not the membranes of origin of X2. Staining of the oligodendroglial cells has not been excluded.

In these experiments, there was staining of the neuropil and a granular type of staining in the cerebellum molecular layer. The staining was similar to that reported by Rostas and Jeffrey (1973) and by Livett *et al.* (1974). These authors suggested that the granular or punctate staining could represent specific reaction of the antiserum with nerve endings. The anti-RBER serum had been absorbed with rat liver and rat kidney homogenates and after absorption the antiserum still reacted strongly with X2, in immunodiffusion analyses.

It is therefore proposed that the most likely interpretation of the results is that X2 is a component of both synaptic membrane and endoplasmic reticulum. While no quantitative experiments were performed for X1, the available evidence indicates that this antigen is also shared by synaptic membrane and endoplasmic reticulum.

The proposal that synaptic membranes and endoplasmic reticulum share antigens is supported by reports in the literature which indicate that the synaptic membrane fraction and the microsomal fraction share antigenic components (Lim and Hsu, 1971; MacPherson *et al.*, 1973). Antigens shared between mitochondria and synaptic membranes have also been reported (Mickey *et al.*, 1971; Herschman *et al.*, 1972; Raiteri and Bertollini, 1974; MacPherson *et al.*, 1973). Similarly,

myelin and synaptic membranes are known to share antigenic components (Raiteri and Bertollini, 1974; Mickey *et al.*, 1971).

In the majority of cases where specific anti-synaptic membrane antigens were reported, the possibility of anti-microsome antibodies was not tested (i.e.: Raiteri *et al.*, 1974; Howe *et al.*, 1977; Mickey *et al.*, 1971). In view of the present findings, this may have been an important omission.

It is not unexpected that synaptic membranes and endoplasmic reticulum would share antigens since a number of components have been demonstrated to be present in both membranes. Thus, acetylcholinesterase (Kokko, 1969; Novikoff, 1967) and the acidic protein, 14-3-2 (Ronnback *et al.*, 1977) have been detected in SM and endoplasmic reticulum.

The synapse is a region of intense activity and may need a continuous supply of certain components from the endoplasmic reticulum. Thus, components such as X1 and X2 could be present in both RSM and RBER. That these antigens appear to be integral components of RSM and RBER lend support to current theories of membrane formation which propose that membrane components are transported, from their site of synthesis in the endoplasmic reticulum of the perikaryon, in vesicles associated with the tubules of the smooth endoplasmic reticulum that connect with the plasma membrane (including SM) (Droz, 1975; Rothman and Lenard, 1977).

PAGE analysis of SM and microsomes support these theories since there is considerable similarity and overlap,

both qualitatively and quantitatively, between the protein constituents of SM and microsomes (Kornguth, 1973; Gurd *et al.*, 1974; Jones *et al.*, 1975; Mahler *et al.*, 1975). This is the case for the RSM and RBER used for the present study (see Figure 10). The molecular weights of the predominant bands detected in Figure 10 correspond quite closely to the molecular weights of the major SM components as reported in the literature (for review see Mahler, 1977). The concentration of acrylamide was chosen so as to obtain good resolution of components with molecular weights corresponding to X2. Proteins with molecular weights above 90,000 daltons remain in the top 0.5 cm of these gels. Since many synaptic membrane proteins with molecular weights ranging from 90,000 to 275,000 daltons have been detected (Mahler, 1977), it is probable that the heavy staining seen at the top of the gels may not be due to undissociated aggregates but may represent proteins of high molecular weight.

It should be mentioned that antigens X1 and X2 could be detected in Triton X-100 extracts of intraterminal mitochondria (subsynaptosomal fraction F_5 in Figure 1) and in purified rat brain myelin; however, X1 and X2 were minor components of these fractions. Therefore, this presentation has been restricted to the study of X1 and X2 in RSM and RBER; although it is recognized that these antigens may be components of other membranes.

It is interesting that treatment of RSM and RBER with Triton X-100 solubilized about the same quantity of X2 from the two membrane preparations (Table 4). Although the amount

of X2 remaining in the Triton residues could not be estimated, the results indicate that RSM and RBER contained the same amount of X2.

Major differences between RSM and RBER Triton extracts could be found only after analysis of acetone precipitates of the Triton extracts. Thus, the RSM-Txla acetone precipitates were not as soluble in water as were the RBER-Txla acetone precipitates. In fact, only 17% of X2 and 33% of the protein present in RSM-Txla acetone precipitates were soluble in water. In contrast, 49% of X2 and 64% of the protein present in RBER-Txla acetone precipitates were soluble in water.

The X2 specific activities, of RBER-Txla and RBER-Tx2 water insoluble acetone precipitates, and the RSM-Tx2 water soluble and water insoluble acetone precipitates, were very high. It can be calculated that X2 has been enriched by about 8-10 fold in these extracts as compared to their respective unprecipitated Triton extracts.

Considerable success was achieved in purifying X2. It will be seen from Figures 21 and 23 that, after gel filtration of RBER-Tx2 extracts, antigen X2 was observed in fractions (containing X2) with only 2 or 3 contaminants. The contaminants correspond to proteins with molecular weights sufficiently different from the estimated molecular weights of X2, so that one would expect to be able to eliminate these contaminants by re-chromatography.

Since antigen X2 was obtained in equal quantity by Triton X-100 extraction of either RSM or RBER, it was decided

to use RBER as the source of X2 because the RSM available represented only one tenth of the RBER. Approximately 16 mg of RSM protein were obtained from each subcellular fractionation and salt washing removed about 45% of the RSM protein leaving 9 mg of washed RSM protein. About 30 mg of washed membrane is required to produce sufficient Tx2 for one gel filtration; thus three subcellular fractionations are required for one gel filtration of RSM-Tx2. In contrast, each subcellular fractionation yields enough RBER-Tx2 for two gel filtration experiments.

It will be seen from Figure 24 that acetone precipitates of RMS-Tx2 have the least number of contaminants and either the water soluble or water insoluble RSM-Tx2 acetone precipitates would be the fractions of choice for purification of X2.

The molecular size of antigen X2 was found to be approximately 180,000 daltons when Triton or butanol extracts are eluted from Sepharose 6B columns, using phosphate buffer as eluant. Using SDS as eluant, the molecular size of X2 was found to be approximately 28,000 daltons. These results indicate that the antigen either exists as an aggregate in Triton extracts or forms part of Triton micelles. The micellar weight of Triton X-100 is approximately 90,000.

By PAGE in the presence of SDS, the molecular size of X2, in a butanol extract of RBER, an RBER-Txla extract and an RBER-Tx2 extract was estimated to be 30,000, 32,000 and 36,000 daltons respectively. This corresponds closely with the molecular weight (28-32,000) calculated for the major component detected in PAGE pattern obtained with acetone

precipitates of RBER and RSM Triton extracts (Figure 26).

Kelly and Cotman (1977a) have reported that a peptide with a molecular weight of 32,000 daltons accounted for 5.3% of the total synaptic complex protein. Another component, with a molecular weight of 28,000 daltons accounted for 7.8% of the total postsynaptic density protein. Similarly, two components with molecular weights approximately 33,000 daltons and 28,000 daltons have been detected in PAGE patterns of synaptic membrane (Blitz and Fine, 1974; Mahler *et al.*, 1975; Wang and Mahler, 1976).

Cotman *et al.* (1971) have reported that synaptic junctional complexes (SJC) can be isolated after treatment of synaptic membranes with Triton X-100. The SJC are preserved under conditions where about 40% of the membrane protein has been solubilized; however, when the Triton/protein ratio is increased, more protein is solubilized and few SJC can be recovered. These results have been confirmed by Bloom and Davis (1973) and by Levitan *et al.* (1972).

In the present report, salt washing removes about 45% of the RSM membrane protein and the Tx1a and Tx1b extracts contain a further 10% of the original RSM protein. At this stage, the remaining residue should consist mainly of SJC's. The Tx2 extracts were found to contain X2 indicating that X2 is possibly a component of SJC. Antigen X2 can even be detected in the final Triton residue, where about 67% of the initial RSM protein has been removed.

CHAPTER 5. CONCLUSIONS

(1) Antigens X1 and X2 were found to be the major immunogens of RSM (subsynaptosomal fraction F_2) and were clearly demonstrated to be identical to the two most prominent antigens of RBER (salt washed microsomal fraction) by immunodiffusion and immunoelectrophoretic analysis.

(2) Antigen X2 can be detected in about equal amounts in Triton X-100 extracts of RSM and RBER.

(3) The results, from immunodiffusion analyses, from quantitative single radial immunodiffusion analyses, and from a study of brain subcellular membrane cross contamination (using marker enzymes for microsomes and plasma membrane), clearly suggest that X1 and X2 are integral components of both synaptic membranes and endoplasmic reticulum; however, the possibility, that either synaptic membranes or endoplasmic reticulum could be the membrane of origin for these antigens, has not completely been eliminated.

(4) The effects of anti-RSM serum, absorbed with either RSM or RBER, on inhibition of synaptosome swelling (in 0.4M glycerol) support the contention that the majority of

antigens present on the synaptosome surface are shared with endoplasmic reticulum. Some (5-10%) specific anti-synaptosome antibody activity was detected. Since swelling of the microsomal fraction was also demonstrated, interpretation of synaptosome swelling inhibition results must be done with caution because the synaptosome fraction may be contaminated with microsomes.

(5) The electrophoretic mobility in agarose at pH 8.6 of X2 from Triton X-100 extracts of RSM and RBER is similar to that of a serum γ globulin run under the same conditions.

(6) Butanol-water treatment of RSM or RBER yields water phases in which antigen X2 exists in two molecular forms, one form being much more basic than the antigen extracted by Triton X-100.

(7) Antigen X2 was extensively purified by gel filtration of RBER-Tx2 extracts. Only two contaminants can be observed in the purest fractions of X2. Since RSM-Tx2 extracts contain less contaminants than RBER-Tx2 extracts, it is expected that X2 can be obtained in pure form by gel filtration of RSM-Tx2 extracts.

(8) It has been estimated that X2 has a molecular weight of 28-32,000 daltons.

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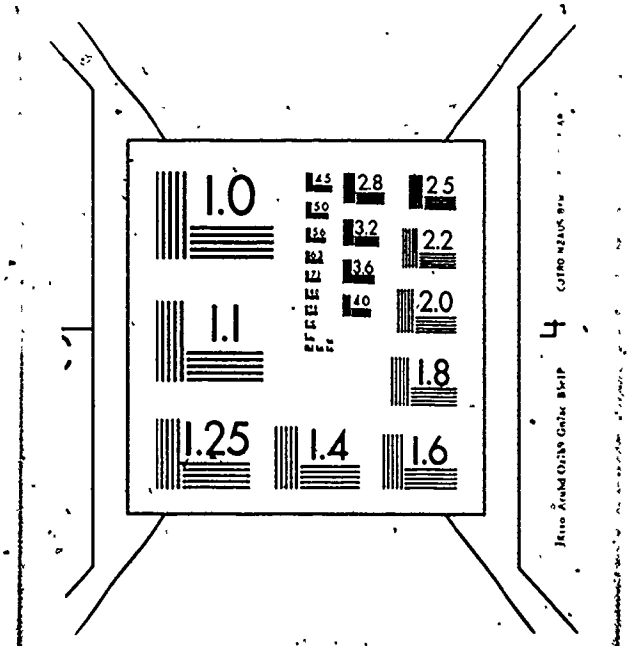

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